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(54) Title: COMPOUNDS, METHODS OF SCREENING AND METHODS OF TREATMENT FOR CENTRAL AND PERIPHERAL NERVOUS SYSTEM DISORDERS

(57) Abstract

The present invention relates to methods and compositions for treating selected conditions of the central and peripheral nervous systems employing non–synaptic mechanisms. More specifically, one aspect of the present invention relates to methods and materials for treating seizure and seizure disorders, epilepsy, status epilepticus, migraine, spreading depression, intracranial hypertension; for treating the pathophysiological effects of head trauma, stroke, ischemia and hypoxia; for treating or protecting from the pathophysiological effects of neurotoxic agents such as ethanol; and for treating neuropsychiatric disorders and central nervous system edema by administering agents that modulate ionic concentrations and/or ionic gradients in the brain, particularly ion–dependent or cation–chloride cotransporter antagonists. Electrolyte cotransport antagonists and combinations of such compositions with other agents for treating various conditions are disclosed. The present invention also relates to methods and compositions for treating pain by administering ion–dependent cotransporter antagonists. Methods and compositions for enhancing cortical function, for example, in centers of cognition, learning and memory, by administering ion–dependant cotransporter agonists are disclosed. Methods and systems for screening drug candidate compounds for desired activities using *in vitro* and *in vivo* systems are disclosed.

COMPOUNDS, METHODS OF SCREENING AND METHODS OF TREATMENT FOR CENTRAL AND PERIPHERAL NERVOUS SYSTEM DISORDERS

## Technical Field of the Invention

The present invention relates to methods and compositions for treating selected conditions of the central and peripheral nervous systems employing non-synaptic mechanisms. More specifically, one aspect of the present invention relates to methods and materials for treating seizures and seizure disorders, epilepsy, status epilepticus, migraine headache, cortical spreading depression, intracranial hypertension, neuropsychiatric disorders, central nervous system edema; for treating or protecting from the pathophysiological effects of toxic agents such as ethanol and certain infectious agents; for treating the pathophysiological effects of head trauma, stroke, ischemia and hypoxia; and for improving certain brain functions, such as cognition, learning and memory; by administering agents that modulate ionic concentrations and ionic balances in the central nervous system. Specific treatment compositions, including loop diuretics, analogs and derivatives of such compositions, as well as combinations of such compositions with other agents for modulating ionic concentrations and gradients, and for treating various conditions, are disclosed. Materials and methods for treating pain by administering agents that modulate ionic concentrations and gradients in the peripheral nervous system are also disclosed. Methods and systems for screening drug candidate compounds for desired activities using *in vitro* and *in vivo* systems are described.

## Background of the Invention

Conventional treatments for neuronal disorders, such as seizure disorders, epilepsy, and the like, target synaptic mechanisms that affect excitatory pathways, such as by modulating the release or activity of neurotransmitters or inhibitors. Conventional treatment agents and regimen for seizure disorders diminish neuronal excitability and inhibit synaptic firing. One serious drawback of this approach is that while seizures are generally localized, the treatment affects (diminishes) neuronal activity indiscriminately. For this reason, there are serious side effects and repeated use of conventional medications may result in unintended deficiencies in normal and desirable brain

functions, such as cognition, learning and memory. More detailed information concerning particular disorders of interest is provided below.

Epilepsy

Epilepsy is characterized by abnormal discharges of cerebral neurons and typically manifested as various types of seizures. Epileptiform activity is identified with spontaneously occurring synchronized discharges of neuronal populations that can be measured using electrophysiological techniques. This synchronized activity, which distinguishes epileptiform from non-epileptiform activity, is referred to as "hypersynchronization" because it describes the state in which individual neurons become increasingly likely to discharge in a time-locked manner with one another.

Epilepsy is one of the most common neurological disorders, affecting about 1% of the population. There are various forms of epilepsy, including idiopathic, symptomatic and cryptogenic. Genetic predisposition is thought to be the predominant etiologic factor in idiopathic epilepsy. Symptomatic epilepsy usually develops as a result of a structural abnormality in the brain.

Status epilepticus is a particularly severe form of seizure, which is manifested as multiple seizures that persist for a significant length of time, or serial seizures without any recovery of consciousness between seizures. The overall mortality rate among adults with status epilepticus is approximately 20 percent. Patients who have a first episode are at substantial risk for future episodes and the development of chronic epilepsy. The frequency of status epilepticus in the United States is approximately 150,000 cases per year, and roughly 55,000 deaths are associated with status epilepticus annually. Acute processes that are associated with status epilepticus include intractable epilepsy, metabolic disturbances (e.g. electrolyte abnormalities, renal failure and sepsis), central nervous system infection (meningitis or encephalitis), stroke, degenerative diseases, head trauma, drug toxicity and hypoxia. The fundamental pathophysiology of status epilepticus involves a failure of mechanisms that normally abort an isolated seizure. This failure can arise from abnormally persistent, excessive excitation or ineffective recruitment of inhibition. Studies have shown that excessive activation of excitatory amino acid receptors can cause prolonged seizures and suggest that excitatory amino acids may play a causative role. Status epilepticus can also be caused by penicillin and

related compounds that antagonize the effects of γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the brain.

One early diagnostic procedure for epilepsy involved the oral administration of large quantities of water together with injections of vasopressin to prevent the accompanying diuresis. This treatment was found to induce seizures in epileptic patients, but rarely in non-epileptic individuals (Garland et al., *Lancet*, 2:566, 1943). Status epilepticus can be blocked in kainic acid-treated rats by intravenous injection of mannitol (Baran et al., *Neuroscience*, 21:679, 1987). This effect is similar to that achieved by intravenous injection of urea in human patients (Carter, *Epilepsia*, 3:198, 1962). The treatment in each of these cases increases the osmolarity of the blood and extracellular fluid, resulting in water efflux from the cells and an increase in extracellular space in the brain. Acetazolamide (ACZ), another diuretic with a different mechanism of action (inhibition of carbonic anhydrase), has been studied experimentally as an anticonvulsant (White et al., *Advance Neurol.*, 44:695, 1986; and Guillaume et al., *Epilepsia*, 32:10, 1991) and used clinically on a limited basis (Tanimukai et al., *Biochem. Pharm.*, 14:961, 1965; and Forsythe et al., *Develop. Med. Child Neurol.*, 23:761, 1981). Although its mechanism of anticonvulsant action has not been determined, ACZ does have a clear effect on the cerebral extracellular space.

Traditional anti-epileptic drugs exert their principal effect through one of three mechanisms: (a) inhibition of repetitive, high-frequency neuronal firing by blocking voltage-dependent sodium channels; (b) potentiation of γ-aminobutyric acid (GABA)-mediated postsynaptic inhibition; and (c) blockade of T-type calcium channels. Phenytoin and carbamazepine are examples of sodium channel antagonists, which exert their effect at the cellular level by reducing or eliminating sustained high-frequency neuronal depolarization while not appreciably affecting regular firing rates of neurons. Barbiturates, such as Phenobarbital and benzodiazepines, act by enhancing GABA-mediated synaptic inhibition. Both classes of compounds increase the hyperpolarization of the postsynaptic membrane, resulting in increased inhibition. Ethosuximide and valporate are examples of drugs that decrease calcium entry into neurons through T-type voltage-dependent calcium channels.

Current anti-epileptic drug therapies exert their pharmacological effects on all brain cells, regardless of their involvement in seizure activity. Common side effects are

over-sedation, dizziness, loss of memory and liver damage. Additionally, 20-30% of epilepsy patients are refractory to current therapy.

Focus on synaptic hyperexcitability has been a guiding principle in basic research on the mechanisms of epileptogenesis and in the design and discovery of new anti-epileptic drugs. One of the shortcomings of this approach is that most current anti-epilepsy drugs exert their influence in an indiscriminate manner, in both the epileptogenic and normal areas in the brain. The compositions of the present invention offer a novel approach to the treatment of seizures, in part because they act via a non-synaptic pathway.

Migraine

Migraine headaches afflict 10-20 % of the U.S. population, with an estimated loss of 64 million workdays annually. Migraine headache is characterized by pulsating head pain that is episodic, unilateral or bilateral, lasting from 4 to 72 hours and often associated with nausea, vomiting and hypersensitivity to light and/or sound. When accompanied by premonitory symptoms, such as visual, sensory, speech or motor symptoms, the headache is referred to as "migraine with aura," formerly known as classic migraine. When not accompanied by such symptoms, the headache is referred to as "migraine without aura," formerly known as common migraine. Both types evidence a strong genetic component, and both are three times more common in women than men.          The precise etiology of migraine has yet to be determined. It is theorized that persons prone to migraine have a reduced threshold for neuronal excitability, possibly due to reduced activity of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). GABA normally inhibits the activity of the neurotransmitters serotonin (5-HT) and glutamate, both of which appear to be involved in migraine attacks. The excitatory neurotransmitter glutamate is implicated in an electrical phenomenon called cortical spreading depression, which can initiate a migraine attack, while serotonin is implicated in vascular changes that occur as the migraine progresses.

Cortical spreading depression (CSD) is characterized by a short burst of intense depolarization in the occipital cortex, followed by a wave of neuronal silence and diminished evoked potentials that advance anteriorly across the surface of the cerebral cortex. Enhanced excitability of the occipital-cortex neurons has been proposed as the basis for CSD. The visual cortex may have a lower threshold for excitability and

5    therefore is most prone to CSD. Mitochondrial disorders, magnesium deficiency and abnormality of presynaptic calcium channels may be responsible for neuronal hyperexcitability (Welch, K.M.A., Pathogenesis of Migraine, *Seminars in Neurobiology*, vol. 17:4, 1997). During a spreading depression event, profound ionic perturbations occur, which include interstitial acidification, extracellular potassium accumulation and

10    redistribution of sodium and chloride ions to intracellular compartments. In addition, prolonged glial swelling occurs as a homeostatic response to altered ionic extracellular fluid composition and interstitial neurotransmitter and fatty acid accumulation. Studies have shown that furosemide inhibits regenerative cortical spreading depression in anaesthetized cats (Read, SJ, et al, *Cephalagia*, 17:826, 1997).

15        A study of eighty-five patients with refractory transformed migraine type of chronic daily headache (CDH) concluded that acute headache exacerbations responded to specific anti-migraine agents such as ergotamine, dihydroergotamine (DHE), and sumatriptan, and addition of agents such as acetazolamide and furosemide, after diagnosis of increased intracranial pressure, resulted in better control of symptoms (Mathew, N.T.

20    et al. Neurology 46:(5), 1226-1230, May 1996). The authors note that these results suggest a link between migraine and idiopathic intracranial hypertension that needs further research.

        Drug therapy is tailored to the severity and frequency of migraine headaches. For occasional attacks, abortive treatment may be indicated, but for attacks occurring two or

25    more times per month, or when attacks greatly impact the patient's daily life, prophylactic therapy may be indicated. Serotonin receptor agonists, such as sumatriptan, have been prescribed for abortive therapy. Serotonin receptor agonists are thought to constrict dilated arteries of the brain and thereby alleviate the associated pain. Side effects associated with this therapy include tingling, dizziness, warm-hot sensation, and

30    injection-site reactions. Intravenous administration is contraindicated as a consequence of the potential for coronary vasopasms. Ergotamine-based drugs are classified as vasoconstrictors that specifically counteract the dilation of some arteries and arterioles, primarily the branches of the external carotid artery. To prevent ergotamine rebound phenomena, ergotamine should not be repeated on the second or third day of a migraine

35    attack. Yet, if the drug is stopped abruptly, the patient will experience a severe rebound

5      headache.    Excessive consumption of ergotamines may cause symptoms of

vasoconstriction, such as cold clammy extremities, and may lead to ergotism.

Drugs used for prophylactic indications include andrenergic beta-blockers such as

propranolol, calcium channel blockers, or low-dose anti-epileptics.  In particular, anti-

epileptic drugs that increase brain levels of GABA, either by increasing GABA synthesis

10     or reducing its breakdown, appear to be effective in preventing migraine in certain

individuals.  In some patients, tricyclic analgesics, such as amitriptline, can be effective.

NMDA receptor antagonist, acting at one of the glutamate receptor subtypes in the brain,

inhibits CSD.   Drugs or substances currently believed to function as weak NMDA

receptor antagonists include dextromethoraphan, magnesium and ketamine.  Intravenous

15     magnesium has been successfully used to abort migraine attacks.


Neurotoxicity

A variety of chemical and biological agents, as well as some infectious agents,

have neurotoxic effects.  A common example is the pathophysiological effect of acute

20     ethanol ingestion.  Episodic ethanol intoxications and withdrawals characteristic of binge

alcoholism result in brain damage.  Animal models designed to mimic the effects of

alcohol in the human have demonstrated that a single dose of ethanol given for 5-10

successive days results in neurodegeneration in the entorhinal cortex, dentate gyrus and

olfactory bulbs, accompanied by cerebrocortical edema and electrolyte ($Na^+$ and $K^+$)

25     accumulation.    As with other neurodegenerative conditions, research has focused

primarily on synaptically based excitotoxic events involving excessive glutamatergic

activity, increased intracellular calcium and decreased γ-aminobutyric acid.  Co-treatment

of brain damage induced by episodic alcohol exposure with an NMDA receptor

antagonist, Non-NMDA receptor and $Ca^{2+}$ channel antagonists with furosemide reduces

30     alcohol-dependent cerebrocortical damage by 75-85%, while preventing brain hydration

and electrolyte elevations (Collins, M., et al, *FASEB*, vol.12 Feb. 1998).  The authors

observed that the results suggest that furosemide and related agents might be useful as

neuroprotective agents in alcohol abuse.

Cognition, learning and memory

The cognitive abilities of mammals are thought to be dependent on cortical processing. It has generally been accepted that the most relevant parameters for describing and understanding cortical function are the spatio-temporal patterns of activity. In particular, long-term potentiation and long-term depression have been implicated in memory and learning and may play a role in cognition. Oscillatory and synchronized activities in the brains of mammals have been correlated with distinct behavioral states.

Synchronization of spontaneous neuronal firing activity is thought to be an important feature of a number of normal and pathophysiological processes in the central nervous system. Examples include synchronized oscillations of population activity such as gamma rhythms in the neocortex, which are thought to be involved in cognition (Singer and Gray, 1995), and theta rhythm in hippocampus, which is thought to play roles in spatial memory and in the induction of synaptic plasticity (Heurta and Lisman 1995; Heurta and Lisman 1996; O'keefe 1993). To date, most research on the processes underlying the generation and maintenance of spontaneous synchronized activity has focused on synaptic mechanisms. However, there is evidence that nonsynaptic mechanisms may also play important roles in the modulation of synchronization in normal and pathological activities in the central nervous system.

Screening of Candidate Compounds and Evaluating Treatment Efficacy

Drug development programs rely on *in vitro* screening assays and subsequent testing in appropriate animal models to evaluate drug candidates prior to conducting clinical trials using human subjects. Screening methods currently used are generally difficult to scale up to provide the high throughput screening necessary to test the numerous candidate compounds generated by traditional and computational means. Moreover, studies involving cell culture systems and animal model responses frequently don't accurately predict the responses and side effects observed during human clinical trials.

Conventional methods for assessing the effects of various agents or physiological activities on biological materials, in both *in vitro* and *in vivo* systems, generally are not highly sensitive or informative. For example, assessment of the effect of a physiological agent, such as a drug, on a population of cells or tissue grown in culture, conventionally

5    provides information relating to the effect of the agent on the cell or tissue population only at specific points in time. Additionally, current assessment techniques generally provide information relating to a single or a small number of parameters. Candidate agents are systematically tested for cytotoxicity, which may be determined as a function of concentration. A population of cells is treated and, at one or several time points

10   following treatment, cell survival is measured. Cytotoxicity assays generally do not provide any information relating to the cause(s) or time course of cell death.

Similarly, agents are frequently evaluated based on their physiological effects, for example, on a particular metabolic function or metabolite. An agent is administered to a population of cells or a tissue sample, and the metabolic function or metabolite of interest

15   is assayed to assess the effect of the agent. This type of assay provides useful information, but it does not provide information relating to the mechanism of action, the effect on other metabolites or metabolic functions, the time course of the physiological effect, general cell or tissue health, or the like.

U.S. Patents 5,902,732 and 5,976,825 disclose methods for screening drug

20   candidate compounds for anti-epileptic activity using glial cells in culture by osomotically shocking glial cells, introducing a drug candidate, and assessing whether the drug candidate is capable of abating changes in glial cell swelling. This patent also discloses a method for screening drug candidate compounds for activity to prevent or treat symptoms of Alzheimer's disease, or to prevent CNS damage resulting from

25   ischemia, by adding a sensitization agent capable of inducing apoptosis and an osmotic stressing agent to CNS cells, adding the drug candidate, and assessing whether the drug candidate is capable of abating cell swelling. A method for determining the viability and health of living cells inside polymeric tissue implants is also disclosed, involving measuring dimensions of living cells inside the polymeric matrix, osmotically shocking

30   the cells, and then assessing changes in cell swelling. Assessment of cell swelling activity is achieved by measuring intrinsic optical signals using an optical detection system.

**Summary of the Invention**

35   Selected treatment compositions and methods of the present invention are useful for treating central nervous system conditions, including seizures and seizure disorders,

5      epilepsy, including Status epilepticus, migraine headaches, cortical spreading depression,
       intracranial hypertension, neuropsychiatric disorders, and central nervous system edema.
       Selected treatment compositions and methods of the present invention are also suitable
       for treating or protecting from the pathophysiological effects of neurotoxic agents such as
       ethanol and certain infectious agents, and for treating the pathophysiological effects of

10     head trauma, stroke, ischemia and hypoxia.   According to another embodiment, treatment
       agents and methods of the present invention improve function in certain cortical tissue,
       such as in cortical centers of cognition, learning and memory.   Additionally, treatment
       agents and methods of the present invention are useful for treating pain by affecting or
       modulating the conduction of impulses associated with pain in the peripheral nervous

15     system.   Treatment compositions and methods of the present invention may be used
       episodically or prophylactically and are suitable for both human and veterinary
       applications.

              Methods and compositions of the present invention involve treatment of various
       conditions of the central and peripheral nervous systems by means of non-synaptic

20     mechanisms and, more specifically, by modulating the synchronization of neuronal
       population activity.   According to a preferred embodiment, the synchronization of
       neuronal population activity is manipulated by modulating anionic concentrations and
       gradients in the central and/or peripheral nervous systems.  Ion dependent cotransporter
       antagonists are suitable treatment compositions, anion cotransporter antagonists are

25     preferred treatment compositions, and cation-chloride cotransporter antagonists are
       especially preferred treatment compositions.  According to one enbodiment, $Na^+,K^+,2Cl^-$
       chloride cotransporter antagonists are especially preferred treatment agents for
       modulating the synchronization of neuronal population activity.  Anion cotransporter
       antagonists are useful for treating conditions such as seizures, epilepsy and status

30     epilepticus, cortical spreading depression and migraine headaches, intracranial
       hypertension, neuropsychiatric disorders, central nervous system edema, for treating or
       protecting from the pathophysiological effects of neurotoxic agents such as ethanol and
       certain infectious agents, and for reducing the perception of pain. Chloride cotransporter
       agonists are preferred treatment agents, and cation-chloride cotransporter agonists are

35     especially preferred treatment agents for improving function in cortical areas associated
       with cognition, learning and memory, for example.  Methods for screening candidate

compounds for ion-dependent cotransporter agonist and antagonist activity, and for efficacy in treating various conditions and disorders are also provided.

Reference to the methods and compositions of the present invention using "non-synaptic" mechanisms means that mechanisms associated with neuronal excitability, such as the release or activity of transmitters, or the release or activity of inhibitors, are not affected by methods and agents of the present invention. Similarly, ion channels and receptors are not directly affected by methods and compositions of the present invention. Rather, the methods and treatment agents of the present invention affect the synchronization, or relative synchrony, neuronal population activity. Preferred methods and treatment agents of the present invention modulate the extracellular anionic chloride concentration and/or the gradients in the central or peripheral nervous system without substantially affecting neuronal excitability.

One aspect of the present invention relates to treatment agents and methods for modulating the synchronization of neuronal discharges by diminishing or eliminating hypersynchronization of neuronal population activity associated with seizures and other pathophysiologies of the central nervous system. In one embodiment, the treatment composition is capable of modulating the anion concentration, preferably the chloride concentration, in the extracellular space in the central nervous system. In a preferred embodiment, the treatment agent is a chloride cotransporter antagonist. In another preferred embodiment, the treatment agent is a cation chloride cotransporter antagonist, and in an especially preferred embodiment, the treatment composition is a glial cell $Na^+,K^+,2Cl^-$ cotransporter antagonist. According to yet another preferred embodiment, the treatment agent has a high level of cation-chloride cotransporter antagonist activity in glial cells, and has a lower level of ion-dependent cotransporter activity in neuronal and kidney cells. Preferred agents for treatment of central nervous system conditions are preferably capable of crossing the blood brain barrier, or are administered using delivery systems that facilitate delivery of agents to the central nervous system.

In general, loop diuretics, such as furosemide, bumetanide, ethacrinic acid, and the like, exhibit ion-dependent cotransporter antagonist activity and are suitable for use as treatment compositions of the present invention. Although such loop diuretics produce the desired modulation of the extracellular anionic chloride concentrations and ionic gradients and, hence, modulation of synchronization of neuronal population activity, they

5      may also produce other, undesired effects. Furosemide, for example, acts as a cation-
chloride cotransporter antagonist in both glial and neuronal cells, as well as in the kidney.
Especially preferred treatment agents of the present invention, exhibiting ion-dependent
cotransporter antagonist activity, exhibit a high degree of activity in glial cell populations,
and exhibit a lesser degree of activity in neuronal and renal cell populations.

10          Methods for treating status epilepticus involve administering an ion-dependent
cotransporter antagonist, preferably a cation-chloride cotransporter antagonist, in
combination with another treatment agent. Furosemide and other loop diuretics are
suitable cation-chloride cotransporter antagonists. Experimental studies have shown that
furosemide treatment produces a transient and early increase in synchronization of

15   neuronal population activity, followed by a persistent and complete disruption of the
hypersynchronization characteristic of epileptiform activity. Treatment of status
epilepticus, according to the present invention, involves administration of an ion-
dependent cotransporter antagonist, preferably a cation-chloride cotransporter
antagonists, such as furosemide, in combination with another agent, such as a barbiturate,

20   that is capable of treating the symptoms associated with the transient and early increase in
synchronization of neuronal population activity observed upon administration of
furosemide.

            Methods and treatment compositions for treating seizures and seizure disorders,
epilepsy, migraine headaches, cortical spreading depression, intracranial hypertension,

25   neuropsychiatric disorders, and for treating or protecting from the pathophysiological
effects of neurotoxic agents, head trauma, stroke, ischemia and hypoxia involve
modulating the synchronization of neuronal population activity, preferably by modulating
ion gradients in the central nervous system. Ion-dependent cotransporter antagonists are
preferred treatment compositions, and cation-chloride cotransporter antagonists are

30   especially preferred treatment compositions. If the ion-dependent cotransporter
antagonist treatment composition has activity, for example, with respect to glial cells, but
has lower or substantially no activity with respect to neuronal cells, it is suitable for
administration alone. If the ion-dependent cotransporter antagonist treatment
composition has activity with respect to neuronal as well as other types of cells, it is

35   preferably administered in combination with another agent, such as conventional anti-
epileptic or anti-convulsant agent.

5          Another aspect of the present invention relates to methods and agents for relieving pain, or the perception of pain, by effecting or modulating propagation of action potentials or conduction of impulses in certain nerve fibers in the peripheral nervous system. More specifically, changes in extracellular ionic concentrations and ionic gradients in cells in the peripheral nervous system, affected by ion-dependent

10      cotransporters, diminishes the perception or sensation of pain. Ion-dependent cotransporter antagonists and, preferably, cation-chloride cotransporter antagonists, delivered to the peripheral nervous system, are preferred treatment compositions for the reduction of pain.

          Yet another aspect of the present invention relates to methods and agents for

15      enhancing the function of cognitive, learning and memory centers in the central nervous system. Enhanced synchronization of neuronal population activity improves function in centers associated with cognitive abilities, learning and memory in central nervous system cortex. Treatment compositions and methods of the present invention for enhancing cognitive, learning and memory functions involve modulating the synchronization and

20      timing of neuronal population activity, preferably by enhancing synchronization and coordinating timing. According to one embodiment, enhancement of synchronization is achieved by administering an agent capable of modulating extracellular anionic chloride concentrations and ionic gradients in the brain. Ion-dependent cotransporter agonists are preferred treatment agents, and cation-chloride cotransporter agonists are especially

25      preferred. Methods for screening candidate compounds for ion-dependent cotransporter agonist activity are also provided.

          Screening methods and systems of the present invention employ optical, or spectroscopic, detection techniques to assess the physiological state of biological materials including cells, tissues, organs, subcellular components and intact organisms.

30      The biological materials may be of human, animal, or plant origin, or they may be derived from any such materials. Static and dynamic changes in the geometrical structure and/or intrinsic optical properties of the biological materials in response to the administration of a physiological challenge or a test agent, are indicative and predictive of changes in the physiological state or health of the biological material.

35          Two different classes of dynamic phenomena are observed in viable biological materials using optical detection techniques: (1) geometrical changes in the diameter,

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5    volume, conformation, intracellular space of individual cells or extracellular space surrounding individual cells; and (2) changes in one or more intrinsic optical properties of individual cells or of cell populations, such as light scattering, reflection, absorption, refraction, diffraction, birefringence, refractive index, Kerr effect, and the like. Both classes of phenomena may be observed statically or dynamically, with or without the aid

10   of a contrast enhancing agent. Geometrical changes may be assessed directly by measuring (or approximating) the geometrical properties of individual cells, or indirectly by observing changes in the optical properties of cells. Changes in optical properties of individual cells or cell populations may be assessed directly using systems of the present invention.

15        Observation and interpretation of geometrical and/or intrinsic optical properties of individual cells or cell populations is achieved in both *in vitro* and *in vivo* systems without altering characteristics of the sample by applying physiologically invasive materials, such as fixatives. Physiologically non-invasive contrast enhancing agents, such as vital dyes, may be used in desired applications to enhance the sensitivity of optical

20   detection techniques. In applications employing contrast enhancing agents, the optical detection techniques are used to assess extrinsic optical properties of the biological materials.

     Detection and analysis of the geometrical and/or intrinsic optical properties of individual cells or sample cell populations provides information permitting classification

25   of the physiological state of individual cells or sample cell populations. Based on analysis of the geometrical and/or optical properties of a sample cell population, the sample may be classified as viable or non-viable, apoptotic, necrotic, proliferating, in a state of activity, inhibition, synchronization, or the like, or in any of a variety of physiological states, all of which produce distinct geometrical and/or optical profiles.

30   The methods and systems of the present invention therefore provide for identification of the physiological state of a sample population and differentiation among various physiological states.

     An important application of the methods and systems of the present invention involves screening cell populations to assess the effect(s) of exposure to various types of

35   test agents and test conditions. The effect of various test agents and conditions may be evaluated on both normal and pathological sample populations. Safety and cytotoxicity

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testing is conducted by exposing a sample population to a test agent or test condition and assessing the physiological state of the sample population using optical techniques at one or more time points following administration of the test agent or test condition. Such testing may be conducted on various sample populations to determine how a test agent or condition affects a desired target sample population, as well as to predict whether a test agent or condition produces physiological side effects on sample populations that are not the target of the test agent or condition.

According to a preferred embodiment, a pathological condition is simulated in biological materials prior to administration of a test agent or test condition to assess the suitability of the test agent or condition for treating the disease state or compromised condition. Exposure of sample populations to a physiological challenge, such as a change in extracellular osmolarity or ion concentration, altered oxygen or nutrient or metabolite conditions, drugs or diagnostic or therapeutic agents, a disturbance in ion homeostasis, electrical stimulation, inflammation, infection with various agents, radiation, and the like, may simulate a pathological state at a cellular or tissue level. Subsequent exposure of the sample populations a test agent or condition and detection and analysis of changes in geometrical and/or optical properties of the sample populations provides information relating to the physiological state of the sample populations produced by the test agent or condition. Screening techniques may be adapted for use with various types of cell sample populations maintained *in vitro* under appropriate cell culture conditions to provide a high throughput, automated screening system. Alternatively, screening techniques may be adapted to examine cell and tissue populations using various animal models to assess the effect of a physiological challenge and/or administration of a test agent on various cell populations in animal models *in situ*. Moreover, because the screening techniques of the present invention are physiologically non-invasive and use spectroscopic techniques, they may be adapted to examine cell and tissue populations in humans, *in situ*, to assess or monitor a patient's condition, and to assess or monitor the efficacy of a treatment agent or regimen.

Changes in geometrical and/or optical properties of individual cells or cell populations may be determined by reference to empirically determined standards for specific cell types, cell densities and various physiological states, or appropriate controls may be run in tandem with the test samples to provide direct comparative data. Data is

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5      collected and, preferably, stored at multiple time points to provide data relating to the

time course of the effect of a test agent or condition on sample populations. Strategies for

designing screening protocols, including appropriate controls, multiple samples for

screening various dosages, activities, and the like, are well known in the art and may be

adapted for use with the methods and systems of the present invention.

10

**Brief Description of the Drawings**

Figs. 1A, 1A1, 1B, 1B1, 1C, 1C1 and 1D show the effect of furosemide on

stimulation evoked after discharge activity in rat hippocampal slices.

Figures 2A - 2R show furosemide blockade of spontaneous epileptiform burst

15     discharges across a spectrum of *in vitro* models.

Figures 3A - 3H show furosemide blockade of kainic acid-evoked electrical

"status epilepticus" in urethane-anesthetized rats, with EKG recordings shown in the

upper traces and cortical EEG recordings shown in the bottom traces.

Figures 4A and 4B show a schematic diagram of ion cotransport under conditions

20     of reduced chloride concentration.


**Detailed Description of the Invention**

Preferred treatment agents and methods of the present invention, for use in

treating seizures and other pathophysiological disorders of the central nervous system,

25     modulate or disrupt the synchrony of neuronal population activity in areas of heightened

synchronization, such as epileptic foci. As described in detail below and illustrated in the

examples, movement of ions and modulation of ionic gradients by means of ion-

dependent cotransporters, preferably cation-chloride dependent cotranporters, is critical to

regulation of neuronal synchronization. Chloride cotransport function has long been

30     thought to be directed primarily to movement of chloride out of cells. The sodium

independent transporter, which has been shown to be neuronally localized, moves

chloride ions out of neurons. Blockade of this transporter, such as by administration of

the loop diuretic furosemide, leads to hyperexcitability, which is the short-term response

to cation-chloride cotransporters such as furosemide. However, the long-term response to

35     furosemide demonstrates that the inward, sodium-dependent movement of chloride ions,

mediated by the glial associated $Na^+,K^+,2Cl^-$ cotransporter, plays an active role in

blocking neuronal synchronization and, hence, seizure, without affecting excitability and stimulus-evoked cellular activity.

According to one embodiment, compositions and methods of the present invention for treating seizures and certain cortical conditions, such as seizures, seizure disorders, epilepsy, including status epilepticus, migraine headaches, cortical spreading depression, intracranial hypertension, neuropsychiatric disorders, central nervous system edema, neural toxicity, such as toxicity induced by alcohol exposure, pathophysiological effects of head trauma, stroke, ischemia and hypoxia, and other conditions resulting from or producing ionic imbalances in the central nervous system, or synchronized discharges of neuronal populations, modulate ion-dependent cotransporter activity. Treatment agents of the present invention for treating seizure disorders and other cortical conditions comprise ion-dependent cotransporter antagonists, preferably cation-chloride cotransporter antagonists. According to a preferred embodiment, agents and methods of the present invention preferentially act on the $Na^+,K^+,2Cl^-$ chloride-dependent cotransport systems of glial cells, and have reduced activity on the chloride-dependent cotransport systems of other cell types, such as neurons and renal cells.

In another embodiment, materials and methods of the present invention are used to treat migraine headache and its precursor condition, cortical spreading depression (CSD). During spreading depression, profound ionic perturbations occur, which include interstitial acidification, extracellular potassium accumulation and redistribution of sodium and chloride ions to intracellular compartments. In addition, prolonged glial swelling occurs as a homeostatic response to altered ionic extracellular fluid composition and interstitial neurotransmitter and fatty acid accumulation. Materials and methods of the present invention inhibit the generation and duration of CSD by blocking the inward sodium-dependent movement of chloride ions mediated by the chloride-dependent cotransporters. Treatment compositions of the present invention for treating migraine headaches and cortical spreading depression comprise cation-chloride cotransporter antagonists. According to a preferred embodiment, agents and methods of the present invention for treating migraine headaches and cortical spreading depression preferentially act on the $Na^+,K^+,2Cl^-$ chloride-dependent cotransport system of glial cells and have reduced activity on the chloride-dependent cotransport systems of other cell types, particularly neurons and renal cells.

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5    Yet another aspect of the present invention involves treatment of neurotoxicity attributable to a variety of chemical and biological agents, as well as some infectious agents. Compositions and methods of the present invention are especially effective in reducing the neurodegenerative effects of acute ethanol ingestion. Additionally, compositions of the present invention may be administered prophylactically to protect

10   cortical tissue from the effects of neurotoxicity attributable, for example, to acute ethanol ingestion. Treatment compositions of the present invention for treating, or for prophylactic administration to protect from neurotoxicity, comprise ion-dependent cotransporter antagonists, preferably cation-chloride cotransporter antagonists. According to a preferred embodiment, agents and methods of the present invention for

15   treating migraine headaches and cortical spreading depression preferentially act on the $Na^+,K^+,2Cl^-$ chloride-dependent cotransport system of glial cells and have reduced activity on the chloride-dependent cotransport systems of other cells types, such as neurons and renal cells.

According to yet another embodiment, compositions and methods of the present

20   invention may be used to alleviate the perception or sensation of pain. In this embodiment, compositions and methods of the present invention affect or modulate the propagation of action potentials in unmyelinated fibers in the peripheral nervous system and, thus, diminish the perception or sensation of pain. Agents of the present invention for treating, or for prophylactic administration to protect from pain, ion-dependent

25   chloride cotransporter antagonists, preferably cation-chloride transporter antagonists, that modulate the extracellular ionic concentration and/or the ionic gradients in the peripheral nervous system. According to a preferred embodiment, compositions of the present invention for treating pain preferentially act on the cation-chloride cotransport system of glial cells, or Schwantz cells, and have reduced activity on the chloride-dependent

30   cotransport systems of other types of cells, such as neurons and renal cells.

In yet another embodiment, materials and methods of the present invention may be used to enhance certain cortical functions, such as cognition, learning and memory. For this application, compositions and methods of the present invention modulate and enhance, rather than disrupt, the synchronization of neuronal population activity.

35   Compositions of the present invention for enhancing cortical function, such as cognition,

learning and memory, comprise ion-dependent cotransporter agonists, preferably cation-chloride cotransporter antagonists.

Compositions of the subject invention are suitable for human and veterinary applications and are preferably delivered as pharmaceutical compositions. Pharmaceutical compositions comprise one or more treatment agents and a physiologically acceptable carrier. Pharmaceutical compositions of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more compounds from the class of chloride cotransporter agonists or antagonists may be combined with another agent, in a treatment combination, and administered according to a treatment regimen of the present invention. Such combinations may be administered as separate compositions, or may be combined for delivery in a complementary delivery system, or may be formulated in a combined composition, such as a mixture or a fusion compound.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the preferred carrier depends upon the preferred mode of administration. Compositions of the present invention may be formulated for any appropriate mode of administration, including for example, topical, oral, nasal, rectal, intravenous, intracranial, spinal tap, intraperitoneal, transdermal, subcutaneous or intramuscular administration. For parenteral administration, such as by subcutaneous injection, the carrier preferably comprises water, saline, glycerin, propylene glycol, alcohol, a fat, a wax and/or a buffer. For oral administration, any of the above carriers, or a solid carrier such as mannitol, lactose, starch, magnesium stearate, sodium lauryl sulphate, lactose, sodium citrate, calcium carbonate, calcium phosphate, silicates, polyethylene glycol, sodium saccharine, talcum, cellulose, glucose, sucrose, dyes, and magnesium carbonate, may be employed. For rectal administration, an aqueous gel formulation, or other suitable formulations that are well known in the art may be administered. Solid compositions may also be employed as fillers in soft and hard filled gelatin capsules. Preferred materials for this include lactose or mild sugar and high molecular weight polyethylene glycols. When aqueous suspensions or elixirs are desired for oral administration, the essential active ingredient therein may be combined with various sweetening or flavoring agents, coloring matter or

5 dyes and, if desired, emulsifying or suspending agents, together with diluents such as water, ethanol, propylene glycol, glycerin and combinations thereof.

Local intracerebral administration, which reduces systemic distribution of the treatment composition(s), may be provided by perfusion via a mechanized delivery system, such as an osmotic pump, or by implantation of a dosage of the treatment

10 composition(s) incorporated in a non-reactive carrier to provide controlled diffusion of the treatment composition over a time course to a circumscribed region of the brain. Other types of time release formulations may also be implemented. Additionally, direct administration into the cerebral spinal fluid via the spinal cord by injection, osmotic pump or other means is preferred for certain applications.

15 The compositions described herein may be administered as part of a sustained release formulation. Such formulations may generally be prepared using well known technology and administered by, for example, oral, rectal or transdermal delivery systems, or by implantation of a formulation or therapeutic device at one or more desired target site(s). Sustained-release formulations may contain a treatment composition

20 comprising an ion-dependent cotransporter agonist or antagonist alone, or in combination with a second treatment agent, dispersed in a carrier matrix and/or contained within a reservoir surrounded by a rate controlling membrane. Carriers for use within such formulations are biocompatible, and may also be biodegradable. According to one embodiment, the sustained release formulation provides a relatively constant level of

25 active composition release. According to another embodiment, the sustained release formulation is contained in a device that may be actuated by the subject or medical personnel, upon onset of certain symptoms, for example, to deliver predetermined dosages of the treatment composition. The amount of the treatment composition contained within a sustained release formulation depends upon the site of implantation,

30 the rate and expected duration of release, and the nature of the condition to be treated or prevented.

Compositions of the present invention for treatment of cortical disorders or conditions, such as seizures, seizure disorders, epilepsy, status epilepticus, migraine, spreading depression, and other conditions characterized by synchronized neuronal

35 population activity, as well as intracranial hypertension, central nervous system edema, neurotoxicity, and the like, are preferably administered using a formulation and a route of

5     administration that facilitates delivery of the treatment composition(s) to the central

nervous system. Treatment compositions, such as ion-dependent cotransporter

antagonists, preferably cation-chloride cotransporter antagonists, may be formulated to

facilitate crossing of the blood brain barrier, or may be co-administered with an agent that

crosses the blood brain barrier. Treatment compositions may be delivered in liposome

10    formulations, for example, that cross the blood brain barrier, or may be co-administered

with other compounds, such as bradykinins, bradykinin analogs or derivatives, or other

compounds, such as SERAPORT, that cross the blood brain barrier. Alternatively,

treatment compositions of the present invention may be delivered using a spinal tap that

places the treatment composition directly in the circulating cerebrospinal fluid. For some

15    treatment conditions, such as chronic epilepsy, episodic seizures, and during some

episodes of spreading depression and migraine headache, there may be transient or

permanent breakdowns of the blood brain barrier and specialized formulation of the

treatment composition to cross the blood brain barrier is not necessary.

Routes and frequency of administration of the therapeutic compositions disclosed

20    herein, as well as dosages, vary according to the indication, and from individual to

individual, and may be readily established using standard techniques. In general,

appropriate dosages and treatment regimen provide the active composition(s) in an

amount sufficient to provide therapeutic and/or prophylactic benefit. Dosages and

treatment regimen may be established by monitoring improved clinical outcomes in

25    treated patients as compared to non-treated patients. A suitable dose is an amount of a

compound that, when administered as described above, produces a therapeutic response

in a patient. Therapeutically effective dosages and treatment regimen will depend on the

condition, the severity of the condition, and the general state of the patient being treated.

Since the pharmacokinetics and pharmacodynamics of the treatment compositions of the

30    present invention vary in different patients, a preferred method for determining a

therapeutically effective dosage in a patient is to gradually escalate the dosage and

monitor the clinical and laboratory indicia. Appropriate dosages and treatment regimen

for treatment of acute episodic conditions, chronic conditions, or prophylaxis will

necessarily vary to accommodate the condition of the patient.

35    In a representative therapeutic treatment regimen, a pharmaceutical preparation of

the present invention is administered alone or, optionally, in combination with a second

5      agent. In combination treatment for seizures and seizure-related disorders, such as epilepsy, treatment compositions of the present invention comprising an ion-dependent cotransporter antagonist, preferably a cation-chloride cotransporter antagonist, is administered in combination with one or more anti-convulsants or anti-epileptic drugs using a delivery system that delivers the treatment composition(s) to the central nervous

10    system. Often the dose of the second agent may be less than the standard dosage as a consequence of the neurophysiological activity of the ion-dependent cotransporter antagonist. Illustrative second agents for treatment in combination with the subject compositions comprising ion-dependent antagonists, include, for example, phenytoin, carbamazepine, barbiturates, phenobarbital, pentobarbital, mephobarbital, trimethadione,

15    mephenytoin, paramethadione, phenthenylate, phenacemide, metharbital, benzchlorpropanmide, phensuximide, primidone, methsuximide, ethotoin, aminoglutethimide, diazepam, clonazepam, clorazepate, fosphenytoin, ethosuximide, valporate, felbamate, gabapentin, lamotrigine, topiramate, vigrabatrin, tiagabine, zonisamide, clobazam, thiopental, midazoplam, propofol, levetiracetam, oxcarbazepine,

20    CCPene, GYK152466 and sumatriptan. As can be readily appreciated, the above-noted compounds are only examples of suitable treatment combinations, and other compounds or similar classes of compounds are also suitable.

         In one preferred embodiment for treating status epilepticus, a treatment composition of the present invention having ion-dependent cotransporter antagonist

25    activity, preferably cation-chloride cotransporter activity, such as furosemide or another loop diuretic, is administered in combination with a traditional anti-seizure agent, such as a barbiturate. In this treatment regimen, the barbiturate, or another anti-seizure agent acts, via synaptic mechanisms, to damp the hyperexcitability of the neuronal population activity and to treat the symptoms of the seizure. The ion-dependent cotransporter

30    antagonist acts, via non-synaptic mechanisms, to damp the hypersynchronization of neuronal population activity in the area of the seizure activity. This combination of treatment compositions may be administered on an emergency basis for treatment of status epilepticus and may be administered using a variety of delivery techniques that deliver the treatment compositions to the central nervous system. The present invention

35    thus contemplates treatment regimen involving administration of a combination of one or more ion-dependent cotransporter antagonist(s), preferably one or more castion-chloride

5    transporter antagonist, with one or more traditional anti-seizure agent(s) selected, for
     example, from one of the following: phenytoin, carbamazepine, barbiturates,
     Phenobarbital, pentobarbital, mephobarbital, trimethadione, mephenytoin,
     paramethadione, phenthenylate, phenacemide, metharbital, benzchlorpropanmide,
     phensuximide, primidone, methsuximide, ethotoin, aminoglutethimide, diazepam,
10   clonazepam, clorazepate, fosphenytoin, ethosuximide, valporate, felbamate, gabapentin,
     lamotrigine, topiramate, vigrabatrin, tiagabine, zonisamide, clobazam, thiopental,
     midazoplam, propofol, levetiracetam, oxcarbazepine, CCPene, GYK152466 and
     sumatriptan. The present invention also contemplates a combination comprising one or
     more chloride cotransporter antagonists with one or more anti-convulsant or anti-seizure
15   agents. According to one embodiment, the combination comprises a preselected dosage
     of one or more anti-convulsant or anti-seizure agents sufficient to reduce
     hyperexcitability for a period of two hours or less, with a preselected dosage of one or
     more anion-dependent cotransporter antagonists sufficient to reduce
     hypersynchronization of neuronal population activity for a period of two hours or more.

20        According to a preferred embodiment, the present invention contemplates a
     container having a combination of preselected dosages of furosemide, or ·another ion-
     dependent cotransporter antagonist, with a barbiturate. The term "container"
     contemplates packets, jars, vials, bottles and other containers for treatment compositions
     in a solid or particulate delivery system, as well as syringes and other liquid containment
25   means, such as various types of bags, vials, bottles, and the like, having contained therein,
     preselected dosages of the combination agents of the present invention. The combination
     may be packaged and administered such that each composition of the combination is
     packaged and administered separately, or, the compositions may be packaged and
     administered as a mixture for simultaneous administration. The present invention also
30   contemplates an emergency or surgical suite in a hospital, clinic, mobile unit, or the like,
     equipped with one or more containers having a combination of preselected dosages of an
     ion-dependent cotransporter antagonist with an anti-convulsant or anti-epileptic agent.

          Treatment compositions of the present invention for treating migraine headaches
     and cortical spreading depression comprise an ion-dependent cotransporter antagonist,
35   preferably a cation-chloride cotransporter antagonist, optionally in combination with one
     or more other therapeutic compositions. The ion-dependent cotransporter antagonist may

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be administered either together or in conjunction with other treatment modalities, or separately, for example at different times or using different delivery techniques. Often the dose of a conventional treatment composition for migraine or spreading depression may be reduced to less than a standard dosage when the treatment is combined with administration of an ion-dependent cotransporter antagonist. The present invention thus contemplates treatment regimen involving administration of a combination of one or more ion-dependent cotransporter antagonist(s) with one or more traditional migraine headache or spreading depression agent(s) selected, for example, from one of the following: serotonin receptor agonists, ergotamine, dihydroergotamine, sumatriptan, propranolol, metoprolol, atenolol, timolol, nadolol, nifeddipine, nimodipine, verapamil, aspirin, ketoprofen, tofenamic acid, mefenamic acid, naproxen, methysergide, paracetamol, clonidine, lisuride, iprazochrome, butalbital, benzodiazepines and divalproex sodium. As can be readily appreciated, the above-noted compounds are only examples of suitable combined treatments and other compounds or similar classes of compounds are equally suitable.

Treatment compositions of the present invention for treating intracranial hypertension, neuropsychiatric disorders, central nervous system edema, and for treating or protecting from neurotoxicity resulting from exposure to neurotoxic agents such as ethanol, infectious agents, and the like, comprise an ion-dependent cotransporter antagonist. The treatment composition may optionally be administered in combination with one or more other therapeutic compositions. Delivery systems providing delivery of the ion-dependent cotransporter antagonist composition to the central nervous system are preferred.

Treatment compositions of the present invention for reducing pain comprise an ion-dependent cotransporter antagonist, preferably a cation-chloride cotransporter antagonist, optionally in combination with one or more other therapeutic compositions. Such treatment compositions preferably do not have the ability to cross the blood brain barrier and circulate in the peripheral nervous system only. Delivery systems providing delivery of the ion-dependent cotransporter antagonist composition to the peripheral nervous system are preferred.

Treatment compositions of the present invention for enhancing cortical function in regions such as cognitive, learning and memory centers comprise an ion-dependent

cotransporter agonists, preferably a cation-chloride cotransporter agonist, optionally in combination with one or more other therapeutic compositions. Suitable delivery systems provide delivery of the ion-dependent cotransporter agonist treatment composition preferentially to the central nervous system, and more preferably to localized cortical centers of cognition, learning and/or memory.

Methods and systems of the present invention may also be used to evaluate candidate compounds and treatment regimen for diagnosis and treatment of various disorders and conditions. Various techniques for generating candidate compounds potentially having the desired ion-dependent cotransporter agonist or antagonist activity may be employed. Candidate compounds may be generated using well known combinatorial chemistry or molecular modeling techniques starting with known ion-dependent cotransporter antagonists, such as loop diuretics, including furosemide, bumetanide, ethacrinic acid, and the like, and related compounds, and modifying those compounds in ways that would be expected to confer the desired activities and specificities. Similarly, candidate chloride cotransporter agonist compounds may be generated using combinatorial chemistry techniques or molecular modeling techniques starting with known ion-dependent cotransporter agonists, and related compounds, and modifying those compounds in ways that would be expected to confer the desired activities and specificities. Methods for screening candidate compounds for desired activities are described below.

According to one embodiment, methods and systems of the present invention acquire and compare data representative of one or more dimensional properties of individual cells or cell samples. Techniques for acquisition, processing and analysis of data relating to optical properties are known and are described, for example, in U.S. Patents 5,438,989, 5,465,718, 5,845,639, 5,699,798, 5,902,732 and 5,976,825, each of which is incorporated by reference herein in its entirety. Acquisition of data relating to dimensional properties of individual cells is described below. Acquisition and analysis of data relating to dimensional properties may be achieved using the same or similar methods and apparatus described with reference to optical properties.

In sparsely populated cell samples, cell areas may be approximated using a single plane of focus. If it is desired to calculate volume, the z-axis (focus) can be automatically adjusted as well. For example, as an automated and controlled stage

5    moves, an optically transparent container containing the sample population is positioned so that a series of data sets for multiple, spatially resolved areas of interest can be acquired, each image being acquired at a predetermined focal plane. The volume for each z-plane can be approximated (see algorithm below) and then the volumes for each z-coordinate added together.

10       General techniques for approximating cell areas and volumes, based on Doughty, S., "Calculating property for solids of revolution," *Machine Design*, pp 184-186, 10 Dec. 1981, are described below. These techniques are based on Green's theorem:

$$\int (Pdx + Qdy) \qquad \iint \left(\frac{\partial Q}{\partial x} - \frac{\partial P}{\partial y}\right) dxdy$$

15

(boundary)                                    (area)

Individual cells are examined using an appropriate magnifying device. Edge
20   detection of cell boundaries is achieved using, for example, a Sobel operator. The boundary is approximated by fitting it to a plurality of straight line segments of "n" line segments by "n" nodes. The integration of the boundary may be taken as "n" line integrals as follows:

25

$$\int_{\Gamma}(\ldots) ds = \int_{X_1,Y_1}^{X_2,Y_2}(\ldots) ds + \int_{X_2,Y_2}^{X_3,Y_3}(\ldots) ds + \ldots + \int_{X_n,Y_n}^{X_1,Y_1}(\ldots) ds$$

30   There are three cases:

Case 1:      a vertical line, $x$ = constant;

Case 2:      a horizontal line, $y$ = constant;

Case 3:      an inclined line, $y = 5_i(x - x_i) + y_i$

35   The area is thus:

$$A = \iint_A \cdot 1 \, dxdy$$

To apply Green's theorem, the integral can be considered in the form of $\delta Q/\delta x$ - $\delta P/\delta y$ and appropriate functions can be devised, *e.g.*, $P(x, y)$ and $Q(x, y)$. For an area calculation, consider $Q(x, y) = 0$ so that $\int Q dy = 0$, and let $P = -y$ so that $\delta P/\delta y = -1$. Then, the area can be calculated as follows:

$$A = \int\int 1\, dxdy = -\int\int \delta P/\delta y \, dxdy$$

$$= \int\int ( 2Q/\delta x - \delta P/\delta y)\, dxdy$$

$$= \int (Pdx + Qdy)$$

(Green's Theorem)

$$= \int Pdx$$

$$= -\int ydx$$

Case 2: $\Delta A = -y_i (x_{i+1} - x_i)$              Case 1 = 0

Case 3: $\Delta A = -[\, \frac{1}{2} 5_i (x^2_{i+1} - x_i^2) + (y_i - 5_i x_i)(x_{i+1} - x_i)]$

$$A = \sum \Delta A$$

For volume calculations, conventional edge detection using, for example, a Sobel operator, can be used to focus through an individual cell, which can be divided into a plurality (n) of individual, planar sections. The volume for each of the "n" sections can be calculated as $\Delta V_i$; $= \Delta z_i$; $\bullet$ $\Delta A$; and the volume of the entire cell can be approximated as: $V = \sum \Delta V_i$. The determination and comparison of cell areas and volumes is preferably accomplished using computer hardware and/or software implementations.

According to another embodiment, methods and systems of the present invention acquire and compare data representative of one or more optical properties of individual cells or areas of interest in cell sample populations. Changes in optical properties that are indicative of physiological activity and that may be detected include, for example, reflection, refraction, diffraction, absorption, scattering, birefringence, refractive index, Kerr effect, and the like. Changes in optical properties are detected directly using photon sensitive elements and, optionally, optical elements that enhance the detected optical properties.

High resolution detection of dynamic geometrical and optical properties indicative of physiological activity may be accomplished without using dyes or other types of contrast enhancing agents according to the methods and apparatus of the present invention, as evidenced by the U.S. Patents incorporated herein by reference. Many of the assessment techniques and apparatus of the present invention are physiologically noninvasive, in that detection and analysis of geometrical and/or intrinsic optical information does not require direct contact of the area of interest with any agents such as dyes, oils, devices, or the like. For particular applications, it may, however, be useful to administer contrast enhancing agents that amplify differences in an optical property being detected as a function of physiological activity prior to acquiring subsequent data and generating a comparison. The use of contrast enhancing agents is described in detail, with reference to optical imaging of tumor and non-tumor tissue, in U.S. Patent 5,465,718 and U.S. Patent 5,438,989, which are incorporated by reference herein in their entireties. Suitable contrast enhancing agents include fluorescent and phosphorescent materials, dyes that bind to cell membranes, optical probes that preferentially accumulate in blood or in the intracellular space, phase resonance dye pairs, and the like. Detectors appropriate for use with such contrast enhancing agents are well known in the art.

Numerous devices for acquiring, processing and displaying data representative of one or more geometrical and/or optical properties of a cell sample population in culture or an area of interest *in situ* in an animal model or human subject may be employed. One preferred device is a camera that acquires images of one or more areas of interest at predetermined time intervals that can be compared to identify areas of changes in geometrical and/or optical properties that indicate physiological activity or dysfunction. The data acquisition device preferably incorporates or is used in conjunction with a device that magnifies the area of interest, such as a microscope. Magnification sufficient to provide resolution of individual cells is preferred. An inverted microscope such as a Nikon Diophot 300 is suitable.

For high throughput screening techniques using cell sample populations maintained under culture conditions, samples in optically transparent containers such as flasks, plates and multi-well plates, may be placed on an automated stage that is controlled and moved in a programmed fashion to permit periodic examination of individual cells or cell populations according to a programmed schedule. For example, a

multi-well culture plate having a plurality of cell samples may be placed on an automated and controllable microscope stage. The stage is controlled by an automated microcontroller so that it automatically moves into position over each culture well. A data set relating to geometrical and/or optical properties of individual cells or a cell population is acquired for each position. In this manner, the system can rapidly and systematically acquire data corresponding to many samples. The physiological environment in selected wells may be altered by exposure to a physiological challenge, test agent or test condition, and the system may continue to automatically acquire data from the same wells in each culture plate at predetermined time intervals following treatment, with data acquired from various treatment wells being compared to data acquired from various control wells or empirically determined controls.

Acquisition of data representative of one or more geometrical and/or optical properties preferably provides high spatial resolution as well, so that geometrical or optical data corresponding to a particular spatial location is acquired at various time intervals for comparison. In this fashion, data acquired from single cells or highly localized areas of interest in cell sample populations is compared to provide reliable and highly-sensitive information concerning the physiological state or condition of the sample population. High spatial resolution is provided, for example, by implementing high resolution cameras and charge coupled devices (CCDs). Apparatus suitable for obtaining such images have been described in the patents incorporated herein by reference.

Various data processing techniques may be advantageously used to assess the data collected in accordance with the present invention. Comparison data may be assessed or presented in a variety of formats. Processing may include averaging or otherwise combining a plurality of data sets to produce control, subsequent and various comparison data sets. Data may be converted from an analog to a digital form for processing, and back to an analog form for display as an image. Alternatively, data may be acquired, processed, analyzed, and output in a digital form.

Data processing may also include amplification of certain signals or portions of a data set (*e.g.*, areas of an image) to enhance the contrast seen in data set comparisons, and to thereby identify cells or cell populations undergoing changes in geometrical and/or optical properties with a high degree of spatial resolution. For example, according to one embodiment, images are processed using a transformation in which image pixel

5    brightness values are remapped to cover a broader dynamic range of values. A "low"
value may be selected and mapped to zero, with all pixel brightness values at or below the
low value set to zero, and a "high" value may be selected and mapped to a selected value,
with all pixel brightness values at or above the high value mapped to the high value.
Pixels having an intermediate brightness value, representing the dynamic changes in
10   brightness indicative of neuronal activity, may be mapped to linearly or logarithmically
increasing brightness values. This type of processing manipulation is frequently referred
to as a "histogram stretch" and can be used according to the present invention to enhance
the contrast of data sets, such as images, representing changes in neuronal activity.

Data processing techniques may also be used to manipulate data sets to provide
15   more accurate combined and comparison data. For example, for *in vivo* applications,
movement, respiration, heartbeat, seizure or reflex activity may shift an area of interest
during data acquisition. It is important that corresponding data points in data sets are
spatially resolved and precisely aligned to provide accurate combined and comparison
data. Optical markers may be fixed at an area of interest and detected as the data is
20   collected to aid in manual alignment or mathematical manipulation of data sets. Various
processing techniques are described below and in the patents incorporated herein by
reference.

Comparison data may be displayed in a variety of ways. Comparison data may be
displayed, for example, in a graphical format that highlights geometrical or optical
25   differences indicative of physiological changes. A preferred technique for presenting and
displaying comparison data is in the form of visual images or photographic frames
corresponding to spatially resolved areas of interest. This format provides a visualizable
spatial location (two- or three-dimensional) of a cell population being analyzed. To
enhance and provide better visualization of high contrast areas indicating changes in
30   geometrical and/or optical properties indicative of physiological activity or dysfunction,
comparison data may be processed to provide an enhanced contrast grey scale or even a
color image. A look up table ("LUT") may be provided, for example, that converts the
grey scale values for each pixel to a different (higher contrast) grey scale value, or to a
color value. Color values may map to a range of grey scale values, or color may be used
35   to distinguish between positive-going and negative-going geometrical or optical changes.

In general, color-converted images provide higher contrast images that highlight changes in optical properties representing physiological activity, function or dysfunction.

Systems of the present invention generally comprise an illumination source for illuminating the biological material, an optical detector for acquiring data relating to a geometrical or optical property of the biological material, and data storage and analysis and output device(s) for storing data relating to a geometrical or optical property of the biological material, comparing various data sets, and/or control data profiles, to generate comparison data relating to changes in geometrical and/or optical properties indicative of changes in the physiological state of sample populations and to provide or display the output data in a useful format.

An emr source is used for illuminating an area of interest during acquisition of data representing one or more dimensional or intrinsic optical properties of cells or tissue at an area of interest. The emr source may be utilized to illuminate an area of interest directly, as when *in vitro* cell cultures maintained in optically transparent containers are illuminated or when tissue is exposed, such as in connection with surgery, or it may be utilized to illuminate an area of interest indirectly through adjacent or overlying tissue such as bone, dura, skin, muscle and the like. The emr source employed in the present invention may be a high or low intensity source, and may provide continuous or non-continuous illumination. Suitable illumination sources include high and intensity sources, broad spectrum and non-chromatic sources, tungsten-halogen lamps, lasers, light emitting diodes, and the like. Cutoff filters for selectively passing all wavelengths above or below a selected wavelength may be employed. A preferred cutoff filter excludes all wavelengths below about 695 nm.

Preferred emr wavelengths for acquiring data relating to intrinsic optical signals include, for example, wavelengths of from about 450 nm to about 2500 nm, and most preferably, wavelengths of the near infrared spectrum of from about 700 nm to about 2500 nm. Generally, longer wavelengths (*e.g.*, approximately 800 nm) are employed to detect cellular or tissue condition of locations beneath the surface of cells or tissue, or beneath other materials such as skin, bone, dura, and the like. cortical activity. Selected wavelengths of emr may also be used, for example, when various types of contrast enhancing agents are administered. The emr source may be directed to the area of interest by any appropriate means. For some applications, the use of optical fibers is preferred.

One preferred arrangement provides an emr source through strands of fiber optic using a beam splitter controlled by a D.C. regulated power supply (Lambda, Inc.).

The optical detection methods of the present invention may also usefully employ non-continuous illumination and detection techniques. For example, short pulse (time domain), pulsed time, and amplitude modulated (frequency domain) illumination sources may be used in conjunction with suitable detectors (*see*, Yodh, A. and Chance, B., *Physics Today*, March, 1995). Frequency domain illumination sources typically comprise an array of multiple source elements, such as laser diodes, with each element modulated at 180° out of phase with respect to adjacent elements (*see*, Chance, B. et al., *Proc. Natl. Acad. Sci. USA*, 90:3423-3427, 1993). Two-dimensional arrays, comprising four or more elements in two orthogonal planes, can be employed to obtain two-dimensional localization information. Such techniques are described in U.S. Patents 4,972,331 and 5,187,672 which are incorporated by reference herein in their entireties.

Time-of-flight and absorbance techniques (Benaron, D.A. and Stevenson, D.K., *Science* 259:1463-1466, 1993) may also be usefully employed in the present invention. In yet another embodiment of the present invention, a scanning laser beam may be used in conjunction with a suitable detector, such as a photomultiplier tube, to obtain high resolution data images, preferably in the form of an area of interest.

Illumination with a part of the infrared spectrum allows for detection of intrinsic optical signals through tissue overlying or adjacent the area of interest, such as dura and skull. One exemplary infrared emr source suitable for detection of intrinsic optical signals through tissue overlying or adjacent the area of interest is a Tunable IR Diode Laser from Laser Photonics, Orlando, FL. When using this range of far infrared wavelengths, the optical detector is preferably provided as an infrared (IR) detector. IR detectors may be constructed from materials such as indium arsenide, germanium and mercury cadmium telluride, and are generally cryogenically cooled to enhance their sensitivity to small changes in infrared radiation. One example of an IR detection system which may be usefully employed in the present invention is an IRC-64 infrared camera (Cincinnati Electronics, Mason, OH).

The area of interest is preferably evenly illuminated to effectively adjust the signal over a full dynamic range, as described below. Nonuniformity of illumination is generally caused by fluctuations of the illumination source and intensity variations

5      resulting from the three-dimensional nature of the tissue surface. More uniform illumination can be provided over the area of interest, for example, by using diffuse lighting, mounting a wavelength cutoff filter in front of the optimal detector and/or emr source, or combinations thereof. Fluctuation of the illumination source itself is preferably prevented by using a light feedback mechanism to regulate the power supply of the

10     illumination source. In addition, a sterile, optically transparent plate may contact and cover an area of interest to provide a flatter, more even contour surface for detection. Fluctuations in illumination can be compensated for using detection processing algorithms, including placing a constant shade grey image marker point at the area of interest as a control point.

15         The system also comprises an optical detector for acquiring a signal representative of one or more optical properties of the area of interest. Any photon detector may be employed as an optical detector. Suitable optical detectors include, for example, photo diodes, photo multiplier tubes, photo sensitive silicon detector chips, such as those provided in CCD devices, and the like. Multiple emr sources and/or multiple photon

20     detectors may be provided and may be arranged in any suitable arrangement. Specialized detectors for detecting selected optical properties may be employed. One preferred optical detector for acquiring data in the format of an analog video signal is a CCD video camera which produces an output video signal at 30 Hz having, for example, 512 horizontal lines per frame using standard RS 170 convention. One suitable device is a

25     CCD-72 Solid State Camera (Dage-MTI Inc., Michigan City, IN). Another suitable device is a COHU 6510 CCD Monochrome Camera with a COHU 6500 electronic control box (COHU Electronics, San Diego, CA). In some cameras, the analog signal is digitized 8-bits deep on an ADI board (analog-to-digital board). The CCD may be cooled, if necessary, to reduce thermal noise.

30         Data processing is an important feature of the optical detection and analysis techniques and systems of the present invention. In use, for example, a CCD apparatus is preferably adjusted (at the level of the analog signal and before digitizing) to amplify the signal and spread the signal across the full possible dynamic range, thereby maximizing the sensitivity of the apparatus. Specific methods for detecting optical signals with

35     sensitivity across a full dynamic range are described in detail in the patents incorporated herein by reference. Means for performing a histogram stretch of the difference frames

(*e.g.,* Histogram/Feature Extractor HF 151-1-V module, Imaging Technology, Woburn, MA) may be provided, for example, to enhance each difference image across its dynamic range. Exemplary linear histogram stretches are described in Green, *Digital Image Processing: A Systems Approach*, Van Nostrand Reinhold: New York, 1983. A histogram stretch takes the brightest pixel, or one with the highest value in the comparison image, and assigns it the maximum value. The lowest pixel value is assigned the minimum value, and every other value in between is assigned a linear value (for a linear histogram stretch) or a logarithmic value (for a log histogram stretch) between the maximum and minimum values. This allows the comparison image to take advantage of the full dynamic range and provide a high contrast image that clearly identifies areas of neuronal activity or inactivity.

Noise (such as 60 Hz noise from A.C. power lines) is filtered out in the control box by an analog filter. Additional adjustments may further enhance, amplify and condition the analog signal from a CCD detector. One means for adjusting the input analog signal is to digitize this signal at video speed (30 Hz), and view the area of interest as a digitized image that is subsequently converted back to analog format.

It is important that data, such as consecutive data sets corresponding to a particular area of interest, be aligned so that data corresponding to the same spatially resolved location is compared. If data sets are misaligned prior to comparison, artifacts are introduced and the resulting comparison data set may amplify noise and edge information. Data misalignment may be caused by sample movement or motion, heartbeat, respiration, and the like. Large movements of cells in an area of interest being analyzed may require a new orientation of the detector. It is possible to compensate for small movements of cells in the area of interest by either mechanical or computational means, or a combination of both.

Real-time motion compensation and geometric transformations may also be used to align corresponding data. Simple mechanical translation of data or more complex (and generally more accurate) geometric transformation techniques can be implemented, depending upon the input data collection rate and amount and type of data processing. For many types of image data, it is possible to compensate by a geometrical compensation which transforms the images by translation in the x-y plane. In order for an algorithm such as this to be feasible, it must be computationally efficient (preferably

implementable in integer arithmetic), memory efficient, and robust with respect to changes in ambient light.

For example, functional control points or numbers can be located in an area of interest and triangulation-type algorithms used to compensate for movements of these control points. Goshtasby ("Piecewise Linear Mapping Functions for Image Registration," *Pattern Recognition* 19:459-66, 1986) describes a method whereby an image is divided into triangular regions using control points. A separate geometrical transformation is applied to each triangular region to spatially register each control point to a corresponding triangular region in a control image.

"Image warping" techniques may be employed whereby each subsequent image is registered geometrically to the averaged control image to compensate for movement. Image warping techniques (described in, for example, Wolberg, *Digital Image Warping*, IEEE Computer Society Press: Los Alamitos, CA, 1990), may be used. Image warping techniques can further indicate when movement has become too great for effective compensation and a new averaged control image must be acquired.

The data storage processing and analysis function is generally performed and controlled by a host computer. The host computer may comprise any general computer (such as an IBM PC type with an Intel 386, 486, Pentium or similar microprocessor or Sun SPARC) that is interfaced with the emr source and/or optical detector and controls data acquisition and flow, comparison computations, analysis, output, and the like. The host computer thus controls acquisition and analysis of data and provides a user interface.

The host computer may comprise a single-board embedded computer with a VME64 interface, or a standard (IEEE 1014-1987) VME interface, depending upon bus band width considerations. Host computer boards which may be employed in the present invention include, for example, Force SPARC/CPU-2E and HP9000 Model 7471. The user interface can be, for example, a Unix/X-Window environment. The image processing board can be, for example, based upon Texas Instruments' MVP and other chips to provide real-time image averaging, registration and other processing necessary to produce high quality difference images for intraoperative viewing. This board will also drive a 120 x 1024 RGB display to show a sequence of difference images over time with pseudo-color mapping to highlight tumor tissue. Preferably, a second monitor is used for the host computer to increase the overall screen real estate and smooth the user interface.

5      The processing board (fully programmable) can support a VME64 master interface to control data transactions with the other boards. Lastly, a peripheral control board can provide electrical interfaces to control mechanical interfaces from the host computer. Such mechanical interfaces can include, for example, the light source and optical detector control box.

10     A real-time data acquisition and display system, for example, may comprise four boards for acquisition, image processing, peripheral control and host computer. A minimal configuration with reduced processing capabilities may comprise just the acquisition and host computer boards. The acquisition board comprises circuitry to perform real-time averaging of incoming video frames and allow readout of averaged

15     frames at a maximum rate bus. A VME bus is preferred because of its high peak bandwidth and compatibility with a multitude of existing VME products. The acquisition board should also support many different types of optical detectors via a variable scan interface. A daughter board may support the interfacing needs of many different types of optical detectors and supply variable scan signals to the acquisition motherboard.

20     Preferably, the unit comprises a daughter board interfacing to an RS-170A video signal to support a wide base of cameras. Other camera types, such as slow scan cameras with a higher spatial/contrast resolution and/or better signal to noise ratio, can be developed and incorporated in the inventive device, as well as improved daughter boards to accommodate such improved cameras.

25     Data relating to dimensional and/or intrinsic optical properties of a sample population acquired, for example, as analog video signals, may be continuously processed using, for example, an image analyzer (e.g., Series 151 Image Processor, Imaging Technologies, Inc., Woburn, MA). An image analyzer receives and digitizes an analog video signal with an analog to digital interface and performs at a frame speed of about

30     1/30th of a second (e.g., 30 Hz or "video speed"). Processing the signal involves first digitizing the signal into a series of pixels or small squares assigned a value (in a binary system) dependent upon the number of photons (i.e., quantity of emr) being reflected off tissue from the part of the area of interest assigned to that pixel. For example, in a standard 512 x 512 image from a CCD camera, there would be 262,144 pixels per image.

35     In an 8 bit system, each pixel is represented by 8 bits corresponding to one of 256 levels of grey.

5          The signal processor may include a programmable look-up table (*e.g.*, CM150-LUT16, Imaging Technology, Woburn, MA) initialized with values for converting grey coded pixel values, representative of a black and white image, to color coded values based upon the intensity of each grey coded value. Using image stretching techniques, the highest and lowest pixel intensity values representing each of the pixels in a digital

10        image frame are determined over a region of the image frame which is to be stretched. Stretching a selected region over a larger range of values permits, for example, easier identification and removal of relatively high, spurious values resulting from noise.

          The signal processor means may further include a plurality of frame buffers having frame storage areas for storing frames of digitized image data received from the

15        A/D interface. The frame storage area comprises at least one megabyte of memory space, and preferably at least 8 megabytes of storage space. An additional 16-bit frame storage area may be provided as an accumulator for storing processed image frames having pixel intensities represented by more than 8 bits. The processor means preferably includes at least three frame buffers, one for storing the averaged control image, another for storing

20        the subsequent image, and a third for storing a comparison image.

          The signal processor may further comprise an arithmetic logic unit (*e.g.*, ALU-150 Pipeline Processor) for performing arithmetical and logical functions on data located in one or more frame buffers. An ALU may, for example, provide image (data) averaging in real time. A newly acquired digitized image may be sent directly to the ALU and

25        combined with control images stored in a frame buffer. A 16 bit result can be processed through an ALU, which will divide this result by a constant (*i.e.*, the total number of images). The output from the ALU may be stored in a frame buffer, further processed, or used as an input and combined with another image.

          Normally, areas of increased physiological activity exhibit an increase of the emr

30        absorption capacity of the cell sample or tissue (*i.e.*, the cell sample gets darker if visible light is used for emr illumination, or an intrinsic signal increases in a positive direction). Similarly, a reduction in physiological activity generally corresponds to a decrease of emr absorption capacity of the tissue (*i.e.*, the tissue appears brighter, or intrinsic signals become negative). For example, data set A is a subsequent averaged image and data set B

35        is an averaged control image. Normally, when a pixel in data set A is subtracted from a pixel in data set B and a negative value results, this value is treated as zero. Hence,

5      difference images cannot account for areas of inhibition. The present invention provides

a method for identifying both negative and positive intrinsic signals, by: (a) subtracting

data set A (a subsequent averaged image) from data set B (an averaged control image) to

create a first difference data set, whereby all negative pixel values are zero; and

(b) subtracting data set B from data set A to create a second difference data set whereby

10     all negative pixel values are zero; and adding the first and second difference data sets to

create a "sum difference data set." The sum difference data set shows areas of increased

activity (*i.e.*, color coded with warmer colors such as yellow, orange, red) and may be

visualized as image areas of less activity or inhibition (*i.e.*, color coded with colder colors

such as green, blue, purple). Alternatively, one can overlay the first difference data set on

15     the second difference data set. The difference output may be visualized as an image and

may be superimposed on the real time analog image to provide an image of the area of

interest (*e.g.*, cortical surface) superimposed with a color-coded difference frame to

indicate where there are intrinsic signals in response to a challenge, stimulus, paradigm,

or the like.

20             The comparison (*e.g.*, difference) data may be further processed to smooth out the

data and remove high frequency noise. For example, a lowpass spatial filter can block

high spatial frequencies and/or low spatial frequencies to remove high frequency noises at

either end of the dynamic range. This provides a smoothed-out processed difference data

set (in digital format). The digitally processed difference data set can be provided as an

25     image and color-coded by assigning a spectrum of colors to differing shades of grey.

This image may then be converted back to an analog image (by an ADI board) and

displayed for a real time visualization of differences between an averaged control image

and subsequent images. Moreover, the processed difference image can be superimposed

over the analog image to display specific tissue sites where a contract enhancing agent

30     may have a faster uptake, or where an intrinsic signal may be occurring.

Processing speed may be enhanced by adding a real time modular processor or

faster CPU chip to the image processor. One example of a real time modular processor

which may be employed in the present invention is a 150 RTMP-150 Real Time Modular

Processor (Imaging Technology, Woburn, MA). The processor may further include an

35     optical disk for storing digital data, a printer for providing a hard copy of the digital

5    and/or analog data and a display, such as a video monitor, to permit the user to continuously monitor the comparison data output.

A single chassis may house all of the modules necessary to provide optical detection and analysis in a format that can be easily interpreted, such as an image format, according to the present invention. The necessary components, whether or to whatever

10   degree integrated, may be installed on a rack that is easily transportable, along with display monitors and peripheral input and output devices.

A preferred high resolution and high performance system comprising a PentaMAX 576x384FT LCD system (by Princeton Instruments Inc., NJ) digitizes the data at the chip and provides a large dynamic range and reduced noise. This system may

15   be interfaced using a PCI-bus to a dual-400 Mhz Pentium PC running windows NT. Image analysis algorithms may be written in C using Microsoft VisualC++ Version 5.0 compiler. For more rapid online processing, the data may be routed to dedicated imaging hardware residing in the PC computer. For example, IM-PCI hardware (by Imaging Technology Inc., Bedford, MA) could be used. One such configuration would consist of

20   the following IM-PCI boards and modules: IM-PCI, AMVS, and a CMALU.

The imaging methods applied to *in vivo* applications may acquire data at the surface of an area of interest. As described in the patents incorporated herein by reference, longer wavelengths of emr (in the infrared range) can be used to image areas of interest which are deeper in tissue or below overlying tissue. In some areas of the body,

25   longer wavelength visible light and near infrared emr can easily pass through such tissue for imaging. Moreover, if a difference image is created between images acquired at two different illumination wavelengths, such as an image acquired at 500 nm emr and an image acquired at 700 nm emr, the difference image represents an optical slice of tissue. Additionally, administration of an imaging agent which absorbs specific wavelengths of

30   emr can act as a filter in the area of interest. In this instance, it is desirable to utilize an imaging agent that remains in the tissue for a prolonged period of time.

In a simple system suitable for assessing cell populations *in situ* in animal or tissue culture models, the systems of the present invention may include one or more optical fiber(s) operably connected to an emr source that illuminates cells or tissue, and

35   another optical fiber operably connected to an optical detector, such as a photodiode, that detects one or more optical properties of the illuminated cells or tissue. The detector may

5      be used to acquire control data representing the "normal" or "background" optical

properties of a sample population, and then to acquire subsequent data representing the

optical properties of the sample population during or following administration of a test

agent or test condition. A physiological challenge and/or a stimulus that stimulates a

disease or pathological state may be administered prior to administration of the treatment

10     agent or condition. The system comprises or is in communication with a data storage and

processing system having information storage and processing capability sufficient to

compare the geometrical and/or optical properties of individual cells or cell samples to

empirically determined standards, or to data acquired at different points in time.

In operation, an area of interest in an *in vitro* or *in vivo* cell sample is illuminated

15     with electromagnetic radiation (emr) and one or a series of data points or data sets

representing one or more geometrical and/or optical properties of a spatially resolved area

of interest is acquired during an interval of "normal" physiological activity. This data

represents a control, or background data profile for that particular cell sample under those

particular physiological conditions. A series of data sets is preferably combined, for

20     example by averaging, to obtain a control data profile. The control data profile is stored

for comparison with other data sets. Similarly, control data sets may be collected and

stored that represent a background data profile for particular cell types under specified

physiological conditions.

Data sets representing the corresponding geometrical and/or optical property of

25     the sample population at the same, spatially resolved areas of interest, are acquired during

a subsequent time period. For monitoring applications, data may be collected at regular

time intervals to monitor the condition of the cell sample and to detect aberrations from

the baseline profile. For screening applications, one or more subsequent data set(s) is

collected during a period following physiological activity or inhibition, induced, for

30     example, by introduction of a test compound or by exposure to a test condition.

Physiological activity or inhibition may be induced by a "natural" occurrence such as a

seizure or stroke in an animal model, or it may be induced by administering a paradigm or

an agent to an *in vitro* or *in vivo* cell sample to stimulate changes in geometrical and/or

optical properties of the cell sample that are indicative of physiological activity or

35     inhibition. During a monitoring interval or stimulation of an intrinsic physiological

response, one or a series of subsequent data sets, representing one or more of the detected

geometrical or optical properties of the area of interest, is acquired. A series of subsequent data sets is preferably combined, for example by averaging, to obtain a subsequent data set. The subsequent data set is compared with the control data set to obtain a comparison data set, preferably a difference data set. Comparison data sets are then analyzed for evidence of changes in geometrical and/or optical properties representative of physiological activity or inhibition within the area of interest.

A cell population may comprise cells in suspension at a sparse cell density, or confluent layers of cells, or layers of cells at other predetermined cell densities, or a tissue sample, such as a tissue slice. Maintenance of a wide variety of cell and tissue samples under cell culture conditions is well known in the art.

The sample population is placed at a predetermined location on a platform, such as on a microscope stage. One exemplary tissue sample is an acute rat hippocampal slice maintained in a submerged perfusion chamber. Alternatively, the sample may be cell samples maintained in cell culture media in flasks, multiple well plates, and the like. Multiple well tissue culture plates may be used for high throughput screening, in combination with an automated stage for positioning cell samples in individual wells for optical detection at predetermined intervals. Programmable, automated positioning devices are well known in the art.

An optical detector (such as a CCD camera) is attached to the camera-port of the microscope. During one or more control period(s) and one or more test period(s), data relating to dimensional and/or optical properties of individual cells or of an area of interest in the cell sample are acquired, stored and processed. Acquisition and processing of data may be accomplished as described below and in the Examples.

The grey-scale image on the upper right is the unprocessed image of the tissue-slice as viewed by the CCD camera. The tissue slice may then be electrically stimulated at two different intensities: a low-intensity electrical stimulus causing a small increase in neuronal and synaptic activation; and a high-intensity electrical stimulus causing a larger increase in neuronal and synaptic activity.

A pseudo-colored image maybe generated as described above. Briefly, an image acquired during the electrical stimulation maybe subtracted from an image acquired in the control state. This image was then filtered with a low-pass filter, histogram-stretched,

5      and pseudo-colored. The colors can be coded to indicate intensity of activity-evoked optical change.

The dynamic optical changes represented in the images produced as described above can also be plotted as a graph. In graphical form, each data-point represents the average change in light-transmission through unite indicated on the pseudo-colored

10     image. A series of images, separated in time intervals, may be acquired and the average value calculated for each image and plotted as a point on the graph. The tissue was electrically stimulated for two seconds at the points indicated by the straight lines. The small peak indicates the maximum optical change induced by the first small electrical stimulation, the larger peak from the second larger stimulus. The electrical stimulation

15     was ceased after two seconds and the tissue was allowed to recover. The plots of the recovery are characteristic of the ion-homeostatic mechanisms of the tissue. Their recovery could be quantified, for example, by finding the best exponential fits for the recovery periods.

Candidate compounds may be screened for chloride cotransporter agonist and/or

20     antagonist activity using screening methods of the present invention with various types of cells in culture such as glial cells, neuronal cells, renal cells, and the like, or *in situ* in animal models. Screening techniques to identify chloride cotransporter antagonist activity, for example, may involve altering the ionic balance of the extracellular space in the tissue culture sample, or *in situ* in an animal model, by producing a higher than

25     "normal" anionic chloride concentration. The geometrical and/or optical properties of the cell or tissue sample subject to this altered ionic balance are determined, and candidate agents are administered. Following administration of the candidate agents, the corresponding geometrical and/or optical properties of the cell or tissue sample are monitored to determine whether the ionic imbalance remains, or whether the cells

30     responded by altering the ionic balances in the extracellular and intracellular space. If the ionic imbalance remains, the candidate agent is likely a chloride cotransporter antagonist. By screening using various types of cells or tissues, candidate compounds having a high level of glial cell chloride cotransporter antagonist activity and having a reduced level of neuronal cell and renal cell chloride cotransporter antagonist activity may be identified.

35     Similarly, effects on different types of cells and tissue systems may be assessed.

5        Additionally, the efficacy of candidate compounds for treating various conditions

of the central and peripheral nervous system may be assessed by simulating or inducing a

condition, such as a seizure, central nervous system edema, ethanol neurotoxicity, cortical

spreading depression, or the like, in a tissue sample or *in situ* in an animal model,

monitoring the geometrical and/or optical properties of the cell or tissue sample during

10      stimulation of the condition, administering the candidate compound, then monitoring the

geometrical and/or optical properties of the cell or tissue sample following administration

of the candidate compound, and comparing the geometrical and/or optical properties of

the cell or tissue sample to determine the effect of the candidate compound. Similarly,

the efficacy of treatment composition(s) in an animal or human subject may be monitored

15      *in situ* using the optical methods and systems of the present invention.

The treatment compositions and methods of the present invention have been

described, above, with respect to certain preferred embodiments. The Examples set forth

below describe the results of specific experiments and are not intended to limit the

invention in any fashion.

20

## Example 1
### The effects of furosemide on epileptiform discharges in hippocampal slices

During these studies, spontaneous epileptiform activity was elicited by a variety of

25      treatments. Sprague-Dawley rats (males and females; 25-35 days old) were decapitated,

the top of the skull was rapidly removed, and the brain chilled with ice-cold oxygenated

slicing medium. The slicing medium was a sucrose-based artificial cerebrospinal fluid

(sACSF) consisting of 220 mM sucrose, 3 mM KCl, 1.25 mM $NaH_2PO_4$, 2 mM $MgSO_4$,

26 mM $NaHCO_3$, 2 mM $CaCl_2$, and 10 mM dextrose (295-305 mOsm). A hemisphere of

30      brain containing hippocampus was blocked and glued (cyanoacrylic adhesive) to the stage

of a Vibroslicer (Frederick Haer, Brunsick, ME). Horizontal or transverse slices 400 $\mu$m

thick were cut in 4° C, oxygenated (95% $O_2$; 5% $CO_2$) slicing medium. The slices were

immediately transferred to a holding chamber where they remained submerged in

oxygenated bathing medium (ACSF) consisting of 124 mM NaCl, 3 mM KCl, 1.25 mM

35      $NaH_2PO_4$, 2 mM $MgSO_4$, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, and 10 mM dextrose (295-305

mOsm). The slice were held at room temperature for at least 45 minutes before being

transferred to submersion-style recording chamber (all other experiments). In the recording chamber, the slices were perfused with oxygenated recording medium at 34-35° C. All animal procedures were conducted in accordance with NIH and University of Washington animal care guidelines.

In most slice experiments, simultaneous extracellular field electrode recordings were obtained from CA1 and CA3 areas. A bipolar tungsten stimulating electrode was placed on the Schaffer collaterals to evoke synaptically-driven field responses in CA1. Stimuli consisted of 100-300 sec duration pulses at an intensity of four times the population-spike threshold. After discharges were evoked by a 2 second train of such stimuli delivered at 60 Hz. Spontaneous interictal-like bursts were observed in slices treated by the following modifications or additions to the bathing medium: 10 mM potassium (6 slices; 4 animals; average – 81 bursts/min.); 200-300 M 4-aminopyridine (4 slices; 2 animals; average – 33 burst/min.); 50-100 M bicuculline (4 slices; 3 animals; average – 14 bursts/min); ) M $Mg^{++}$ (1 hour of perfusion – 3 slices; 2 animals; average – 20 bursts/min. or 3 hours of perfusion – 2 slices; 2 animals); zero calcium/6 mM KCI and 2 mM EGTA (4 slices; 3 animals). In all treatments, furosemide was added to the recording medium once a consistent level of bursting was established.

In the first of these procedures, episodes of after discharges were evoked by electrical stimulation of the Schaffer collaterals (Stasheff et al., *Brain Res.* 344:296, 1985) and the extracellular field response was monitored in the CA1 pyramidal cell region (13 slices; 8 animals). The concentration of $Mg^{++}$ in the bathing medium was reduced to 0.9 mM and after discharges were evoked by stimulation at 60 Hz for 2 seconds at an intensity 4 times the population spike threshold (population spike threshold intensity varied between 20-150 μA at 100-300 μsec pulse duration). The tissue was allowed to recover for 10 minutes between stimulation trials. In each experiment, the initial response of CA1 to synaptic input was first tested by recording the field potential evoked by a single stimulus pulse. In the control condition, Schaffer collateral stimulation evoked a single population spike (Fig. 1A, inset). Tetanic stimulation evoked approximately 30 seconds after discharge (Fig. 1A, left) associated with a large change in intrinsic signal (Fig. 1A, right).

For imaging of intrinsic optical signals, the tissue was placed in a perfusion chamber located on the stage of an upright microscope and illuminated with a beam of

5  white light (tungsten filament light and lens system – Dedo Inc.) directed through the

microscope condenser. The light was controlled and regulated (power supply – Lamda

Inc.) to minimize fluctuations and filtered (695 nm longpass) so that the slice was

transilluminated with long wavelengths (red). Field of view and magnification were

determined by the choice of microscope objectives (4X for monitoring the entire slice).

10  Image-frames were acquired with a charge-coupled device (CCD) camera (Dage MTI

Inc.) at 30 HZ and were digitized at 8 bits with a spatial resolution of 512 x 480 pixels

using an Imaging Technology Inc. Series 151 imaging system; gains and offsets of the

camera-control box and the A/D board were adjusted to optimize the sensitivity of the

system. Imaging hardware was controlled by a 486-PC compatible computer. To

15  increase signal/noise, an averaged-image was composed from 16 individual image-

frames, integrated over 0.5 sec and averaged together. An experimental series typically

involved the continuous acquisition of a series of averaged-images over a several minute

time period; at least 10 of these averaged-images were acquired as control-images prior o

stimulation. Pseudocoloured images were calculated by subtracting the first control-

20  image from subsequently acquired images and assigning a color lookup table to the pixel

values. For these images, usually a linear low-pass filter was used to remove high

frequency noise and a linear-histogram stretch was used to map the pixel values over the

dynamic range of the system. All operations on these images were linear so that

quantitative information was preserved. Noise was defined as the maximum standard

25  deviation of fluctuations of AR/R of the sequence of control images within a given

acquisition series, where AR/R represented the magnitude of the change in light-

transmission through the tissue. Delta R/R was calculated by taking all the difference-

images and dividing by the first control image: (subsequent image – first-control-

image)/first-control-image. The noise was always <0.01 for each of the chosen image

30  sequences. The absolute change in light transmission through the tissue was estimated

during some experiments by acquiring images after placing neutral density filters between

the camera and the light source. On average, the camera electronics and imaging system

electronics amplified the signal 10-fold prior to digitization so that the peak absolute

changes in light transmission through the tissue were usually between 1% and 2%.

35  The gray-scale photo shown in Fig. 1D is a video image of a typical hippocampal

slice in the recording chamber. The fine gold-wire mesh that was used to hold the tissue

5     in place can be seen as dark lines running diagonally across the slice. A stimulating electrode can be seen in the upper right on the stratum radiatum of CA1. The recording electrode (too thin to be seen in the photo) was inserted at the point indicated by the white arrow. Fig. 1A illustrates that two seconds of stimulation at 60 Hz elicited after discharge activity and shows a typical after discharge episode recorded by the extracellular

10    electrode. The inset of Fig. 1A shows the CA1 field response to a single 200 sec test pulse (artifact at arrow) delivered to the Schaffer collaterals. Fig. 1A1 shows a map of the peak change in optical transmission through the tissue evoked by Schaffer collateral stimulation. The region of maximum optical change corresponds to the apical and basal dendritic regions of CA1 on either side of the stimulating electrode. Fig. 1B illustrates

15    sample traces showing responses to stimulation after 20 minutes of perfusion with medium containing 2.5 mM furosemide. Both the electrical after discharge activity (shown in Fig. 1B) and the stimulation-evoked optical changes (shown in Fig. 1B1) were blocked. However, there was a hyper-excitable field response (multiple population spikes) to the test pulse (inset). Figs 1C and 1C1 illustrate that restoration of initial

20    response patterns was seen after 45 minutes of perfusion with normal bathing medium.

        The opposing effects of furosemide-blockade of the stimulation-evoked after discharges and a concomitant increase of the synaptic response to a test-pulse illustrate the two key results: (1) furosemide blocked epileptiform activity, and (2) synchronization (as reflected by spontaneous epileptiform activity) and excitability (as reflected by the

25    response to a single synaptic input) were dissociated. Experiments in which the dose-dependency of furosemide was examined determined that a minimum concentration of 1.25 mM was required to block both the after discharges and optical changes.

## Example 2

### The effects of furosemide on epileptiform discharges in hippocampal slices
30
### perfused with high-K$^+$ (10 mM) bathing medium

        Rat hippocampal slices, prepared as described above, were perfused with a high-K$^+$ solution until extended periods of spontaneous interictal-like bursting were recorded

35    simultaneously in CA3 (top traces) and CA1 (lower traces) pyramidal cell regions (Figs. 2A and 2B). After 15 minutes of perfusion with furosemide-containing medium (2.5 mM

5     furosemide), the burst discharges increased in magnitude (Figs. 2C and 2D). However,

after 45 minutes of furosemide perfusion, the bursts were blocked in a reversible manner

(Figs 2E, 2F, 2G and 2H). During this entire sequence of furosemide perfusion, the

synaptic response to a single test pulse delivered to the Schaffer colalterals was either

unchanged or enhanced (data not shown). It is possible that the initial increase in

10    discharge amplitude reflected a furosemide-induced decrease in inhibition (Misgeld et al.,

*Science* 232:1413, 1986; Thompson et al., *J. Neurophysiol.* 60:105, 1988; Thompson and

Gähwiler, *J. Neuropysiol.* 61:512, 1989; and Pearce, *Neuron* 10:189, 1993). It has

previously been reported (Pearce, *Neuron* 10:189, 1993) that furosemide blocks a

component of the inhibitory currents in hippocampal slices with a latency (<15 min.)

15    similar to the time to onset of the increased excitability observed here. The longer latency

required for the furosemide-block of the spontaneous bursting might correspond to

additional time required for a sufficient block of the furosemide-sensitive cellular volume

regulation mechanisms under high-$K^+$ conditions.

         After testing the effects of furosemide on slices perfused with high-$K^+$, similar

20    studies were performed with a variety of other commonly studied *in vitro* models of

epileptiform discharge (Galvan et al., *Brain Res.* 241:75, 1982; Schwartzkroin and Prince,

*Brain Res.183:61*, 1980; Anderson et al., *Brain Res.* 398:215, 1986; and Zhang et al.,

*Epilepsy Res.* 20:105, 1995). After prolonged exposure (2-3 hours) to magnesium-free

medium (0-$Mg^{++}$), slices have been shown to develop epileptiform discharges that are

25    resistant to common clinically used anticonvulsant drugs (Zhang et al., *Epilepsy Res.*

20:105, 1995). Recordings from entorhinal cortex (Fig. 2I) and subiculum (not shown)

showed that after 3 hours of perfusion with 0-$Mg^{++}$ medium, slices developed bursting

patterns that appeared similar to these previously described "anticonvulsant resistant"

bursts. One hour after the addition of furosemide to the bathing medium, these bursts

30    were blocked (Fig. 2J). Furosemide also blocked spontaneous burst discharges observed

with the following additions/modifications to the bathing medium: (1) addition of 200-

300 μM 4-aminopyridine (4-AP; a potassium channel blocker) (Figs. 2K and 2L); (2)

addition of the GABA antagonist, bicuculline, at 50-100 μM (Figs. 2M ad 2N); (3)

removal of magnesium (0-$Mg^{++}$) – 1 hours perfusion (Figs. 2O and 2P); and (4) removal

35    of calcium plus extracellular chelation (0-$Ca^{++}$) (Figs. 2Q and 2R). With each of these

manipulations, spontaneous interictal-like patterns were simultaneously recorded from

5    CA1 and CA3 subfields (Figs. 2K, 2L, 2M and 2N show only the CA3 trace and Figs. 2O,

2P, 2Q, and 2R show only the CA1 trace). In the $0\text{-}Ca^{++}$ experiments, 5 mM furosemide

blocked the bursting with a latency of 15-20 minutes. For all other protocols, bursting

was blocked by 2.5 mM furosemide with a latency of 20-60 minutes. Furosemide

reversibly blocked the spontaneous bursting activity in both CA1 and CA3 in all

10   experiments (Figs 2L, 2N, 2P and 2R).


## Example 3

### The effects of furosemide on epileptiform activity induced by i.v. injection of kainic acid in anesthetized rats

15

         This example illustrates an *in vitro* model in which epileptiform activity was

induced by i.v. injection of kainic acid (KA) into anesthetized rats (Lothman et al.,

*Neurology* 31:806, 1981). The results are illustrated in Figs. 3A - 3H. Sprague-Dawley

rats (4 animals; weights 250-270 g) were anesthetized with urethane (1.25 g/kg i.p.) and

20   anesthesia maintained by additional urethane injections (0.25 g/kg i.p.) as needed. Body

temperature was monitored using a rectal temperature probe and maintained at 35-37° C

with a heating pad; heart rate (EKG) was continuously monitored. The jugular vein was

cannulated on one side for intravenous drug administration. Rats were placed in a Kopf

stereotaxic device (with the top of the skull level), and a bipolar stainless-steel

25   microelectrode insulated to 0.5 mm of the tip was inserted to a depth of 0.5-1.2 mm from

the cortical surface to record electroencephalographic (EEG) activity in the fronto-parietal

cortex. In some experiments, a 2M NaCl-containing pipette was lowered to a depth of

2.5-3.0 mm to record hippocampal EEG. Data were stored on VHS videotape and

analyzed off-line.

30       Following the surgical preparation and electrode placement, animals were allowed

to recover for 30 minutes before the experiments were initiated with an injection of kainic

acid (10-12 mg/kg i.v.). Intense seizure activity, an increased heart rate, and rapid

movements of the vibrissae were induced with a latency of about 30 minutes. Once stable

electrical seizure was evident, furosemide was delivered in 20 mg/kg boluses every 30

35   minutes to a total of 3 injections. Experiments were terminated with the intravenous

administration of urethane.    Animal care was in accordance with NIH guidelines and approved by the University of Washington Animal Care Committee.

Figs. 3A-3H show furosemide blockade of kainic acid-evoked electrical "status epilepticus" in urethane-anesthetized rats.  EKG recordings are shown as the top traces and EEG recordings are shown as the bottom traces.  In this model,  intense electrical discharge (electrical "status epilepticus") was recorded from the cortex (or from depth hippocampal electrodes) 30-60 minutes after KA injection (10-12 mg/kg) (Figs. 3C and 3D).    Control experiments (and previous reports, Lothman et al., *Neurology*, 31:806, 1981) showed that this status-like activity was maintained for well over 3 hours.  Subsequent intravenous injections of furosemide (cumulative dose: 40-60 mg/kg) blocked seizure activity with a latency of 30-45 minutes, often producing a relatively flat EEG (Figs. 3E, 3F, 3G and 3H).  Even 90 minutes after the furosemide injection, cortical activity remained near normal baseline levels (*i.e.*, that observed prior to the KA and furosemide injections).  Studies on the pharmacokinetics of furosemide in the rat indicate that the dosages used in this example were well below toxic levels (Hammarlund and Paalzow, *Biopharmaceutics Drug Disposition*, 3:345, 1982).

Experimental methods for Examples 4 - 7

Hippocampal slices were prepared from Sprague-Dawley adult rats as described previously.  Transverse hippocampal slices 100 µm thick were cut with a vibrating cutter.  Slices typically contained the entire hippocampus and subiculum.  After cutting, slices were stored in an oxygenated holding chamber at room temperature for at least one hour before recording.  All recordings were acquired in an interface type chamber with oxygenated (95% $O_2$, 5%$CO_2$) artificial cerebral spinal fluid (ACSF) at 34°-35° C.  Normal ACSF contained (in mmol/l): 124 NaCl, 3 KCl, 1.25 $NaH_2PO_4$, 1.2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 dextrose.

Sharp-electrodes for intracellular recordings from CA1 and CA3 pyramidal cells were filled with 4 M potassium acetate.  Field recordings from the CA1 and CA3 cell body layers were acquired with low-resistance glass electrodes filled with 2 M NaCl.  For stimulation of the Schaffer collateral or hilar pathways, a small monopolar tungsten electrode was placed on the surface of the slice.  Spontaneous and stimulation-evoked activities from field and intracellular recordings were digitized (Neurocorder, Neurodata

Instruments, New York, NY) and stored on videotape. AxoScope software (Axon Instruments) on a personal computer was used for off-line analysis of data.

In some experiments, normal or low-chloride medium was used containing bicuculline (20 μM), 4-amino pyridine (4-AP) (100 μM), or high-$K^+$ (7.5 or 12 mM). In all experiments, low-chloride solutions (7, and 21 mM [$Cl^-$]o) were prepared by equimolar replacement of NaCl with $Na^+$-gluconate (Sigma). All solutions were prepared so that they had a pH of approximately 7.4 and an osmolarity of 290-300 mOsm at 35°C and at equilibrium from carboxygenation with 95%$O_2$ / 5%$CO_2$.

After placement in the interface chamber, slices were superfused at approximately 1 ml/min. At this flow-rate, it took 8-10 minutes for changes in the perfusion media to be completed. All of the times reported here have taken this delay into account and have an error of approximately ± 2 minutes.

## Example 4
### Timing of cessation of spontaneous epileptiform bursting in areas in CA1 and CA3

The relative contributions of the factors that modulate synchronized activity vary between areas CA1 and CA3. These factors include differences in the local circuitry and region-specific differences in cell packing and volume fraction of the extracellular spaces. If the anti-epileptic effects of anion or chloride-cotransport antagonism are due to a desynchronization in the timing of neuronal discharge, chloride-cotransport blockade might be expected to differentially affect areas CA1 and CA3. To test this, a series of experiments was performed to characterize differences in the timing of the blockade of spontaneous epileptiform activity in areas CA1 and CA3.

Field activity was recorded simultaneously in areas CA1 and CA3 (approximately midway between the most proximal and distal extent the CA3 region), and spontaneous bursting was induced by treatment with high-[$K^+$]o (12 μM; n = 12), bicuculline (20 mM; n = 12), or 4-AP (100 μM; n = 5). Single electrical stimuli were delivered to the Schaffer collaterals, midway between areas CA1 and CA3, every 30 seconds so that the field responses in areas CA1 and CA3 could be monitored throughout the duration of each experiment. In all experiments, at least 20 minutes of continuous spontaneous

5      epileptiform bursting was observed prior to switching to low [Cl⁻]o (21 mM) or
       furosemide-containing (2.5 mM) medium.

              In all cases, after 30-40 minutes exposure to furosemide or low-chloride medium,
       spontaneous bursting ceased in area CA1 before the bursting ceased in area CA3.  The
       temporal sequence of events typically observed included an initial increase in burst
10     frequency and amplitude of the spontaneous field events, then a reduction in the
       amplitude of the burst discharges which was more rapid in CA1 than in CA3.  After CA1
       became silent, CA3 continued to discharge for 5-10 minutes, until it too no longer
       exhibited spontaneous epileptiform events.

              This temporal pattern of burst cessation was observed with all epileptiform-
15     inducing treatments tested, regardless of whether the agent used for blockade of
       spontaneous bursting was furosemide or low-[Cl⁻]o medium.  Throughout all stages of
       these experiments, stimulation of the Schaffer collaterals evoked hyperexcited field
       responses in both the CA1 and CA3 cell body layers.  Immediately after spontaneous
       bursting was blocked in both areas CA1 and CA3, hyperexcited population spikes could
20     still be evoked.

              We considered the possibility that the observed cessation of bursting in CA1 prior
       to CA3 was an artifact of the organization of synapatic contacts between these areas
       relative to our choice of recording sites.  It is known that the projections of the various
       subregions of CA3 terminate in an organized fashion in CA1; CA3 cells closer to the
25     dentate gyrus (proximal CA3) tend to project most heavily to the distal portions of CA1
       (near the subicular border), whereas CA3 projections arising from cells located more
       distally in CA3 terminate more heavily in portions of CA1 located closer to the CA2
       border.  If the cessation of bursting occurs in the different subregions of CA3 at different
       times, the results of the above set of experiments might arise not as a difference between
30     CA1 and CA3, but rather as a function of variability in bursting activity across CA3
       subregions.  We tested this possibility in three experiments.  Immediately after the
       spontaneous bursting ceased in CA1, we surveyed the CA3 field with a recording
       electrode.  Recordings form several different CA3 locations (from the most proximal to
       the most distal portions of CA3), showed that all subregions of area CA3 were
35     spontaneously bursting during this time that CA1 was silent.

5        The observation that CA3 continued to discharge spontaneously after CA1 became silent was unexpected since population discharges in CA3 are generally thought to evoke discharges in CA1 through excitatory synaptic transmission. As previously described, single-pulse stimuli delivered to the Schaffer collaterals still evoked multiple population spikes in CA1 even after the blockade of spontaneous bursting; thus,

10     hyperexcited excitatory synaptic transmissions in CA3-to-CA1 synapse was intact. Given this maintained efficacy of synaptic transmission, and the continued spontaneous field discharges in CA3, we postulated that the loss of spontaneous bursting in CA1 was due to a decrease in synchronization of incoming excitatory drive. Further, since spontaneous epileptiform discharge in CA3 also eventually ceased, perhaps this desynchronization

15     process occurred at different times in the two hippocampal subfields.


## Example 5

### Effect of chloride-cotransport antagonism on the synchronization of CA1 and CA3 field population discharges

20

         The observation from Example 4 suggested a temporal relationship between the exposure time to low-$[Cl^-]_o$ or furosemide-containing medium and the characteristics of the spontaneous burst activity. Further, this relationship was different between areas CA1 and CA3. In order to better characterize the temporal relationships, we compared the

25     occurrences of CA1 action potentials and the population spike events in the field responses of CA1 and CA3 subfields during spontaneous and stimulation-evoked burst discharge.

         Intracellular recordings were obtained from CA1 pyramidal cells, with the intracellular electrode placed close ($<100$ $\mu M$) to the CA1 field electrode. The slice was

30     stimulated every 20 seconds with single stimuli delivered to the Schaffer collaterals. After continuous spontaneous bursting was established for at least 20 minutes, the bathing medium was switched to bicuculline-containing low-$[Cl^-]_o$ (21 mM) medium. After approximately 20 minutes, the burst frequency and amplitude was at its greatest. Simultaneous field and intracellular recordings during this time showed that the CA1 field

35     and intracellular recordings were closely synchronized with the CA3 field discharges. During each spontaneous discharge, the CA3 field response preceded the CA1 discharge

5   by several milliseconds. During stimulation-evoked events, action potential discharges of
the CA1 pyramidal cell were closely synchronized to both CA3 and CA1 field discharges.

With continued exposure to low-[Cl⁻]o medium, the latency between the
spontaneous discharges of areas CA1 and CA3 increased, with a maximum latency of 30-
40 milliseconds occurring after 30-40 minutes exposure to the bicuculline-containing
10  low-chloride medium. During this time, the amplitude of both the CA1 and CA3
spontaneous field discharges decreased. Stimulation-evoked discharges during this time
closely mimicked the spontaneously occurring discharges in morphology and relative
latency. However, the initial stimulus-evoked depolarization of the neuron (presumably,
the monosynaptic EPSP) began without any significant increase in latency. The time
15  interval during which these data were acquired corresponds to the time immediately prior
to the cessation of spontaneous bursting in CA1.

After 40-50 minutes perfusion with low-[Cl⁻]o medium, the spontaneous bursts
were nearly abolished in CA1 but were unaffected in CA3. Schaffer collateral
stimulation during this time showed that monosynaptically-triggered responses of CA1
20  pyramidal cells occurred without any significant increase in latency, but that stimulation-
evoked field responses were almost abolished. The time interval during which these data
were acquired corresponds to the moments immediately prior to the cessation of
spontaneous bursting in CA3.

After prolonged exposure to low-[Cl⁻]o medium, large increases (>30
25  milliseconds) developed in the latency between Schaffer collateral stimulation and the
consequent CA3 field discharge. Eventually, no field responses could be evoked by
Schaffer collateral stimulation in either areas CA1 and CA3. However, action potential
discharge from CA1 pyramidal cells in response to Schaffer collateral stimulation could
be evoked with little change in response latency. Indeed, for the entire duration of the
30  experiments (greater than two hours), action potential discharges form CA1 pyramidal
cells could be evoked at short latency by Schaffer collateral stimulation. Further,
although stimulation-evoked hyperexcited discharges of CA3 were eventually blocked
after prolonged exposure to low-[Cl⁻]o medium, the antidromic response in CA3 appeared
to be preserved.

35

Example 6

Effects of chloride-cotransport antagonism on the synchronization of burst discharges in
CA1 pyramidal cells


The foregoing data suggest the disappearance of the field responses may be due to a desynchronization of the occurrence of action potentials among neurons. That is, although synaptically-driven excitation of CA1 pyramidal cells was not preserved, action potential synchrony among the CA1 neuronal population was not sufficient to summate into a measurable DC field response. In order to test this, paired intracellular recordings of CA1 pyramidal cells were acquired simultaneously with CA1 field responses. In these experiments, both the intracellular electrodes and the field recording electrodes were placed within 200 μm of one another.

During the period of maximum spontaneous activity induced by bicuculline-containing low-$[Cl^-]_o$ medium, recordings showed that action potentials between pairs of CA1 neurons and the CA1 field discharges were tightly synchronized both during spontaneous and stimulation-evoked discharges. After continued exposure to low-$[Cl^-]_o$ medium, when the amplitude of the CA1 field discharge began to broaden and diminish, both spontaneous and stimulation-evoked discharges showed a desynchronization in the timing of the occurrences of action potentials between pairs of CA1 neurons, and between the action potentials and the field responses. This desynchronization was coincident with the suppression of CA1 field amplitude. By the time that spontaneous bursting in CA1 ceased, a significant increase in latency had developed between Schaffer collateral stimulation and CA1 field discharge. At this time, paired intracellular recordings showed a dramatic desynchronization in the timing of action potential discharge between pairs of neurons and between the occurrence of action potentials and the field discharges evoked by Schaffer collateral stimulation.

It is possible that the observed desynchronization of CA1 action potential discharge is due to the randomization of mechanisms necessary for synaptically-driven action potential generation, such as a disruption in the timing of synaptic release or random conduction failures at neuronal processes. If this were the case, then one would expect that the occurrence of action potentials between a given pair of neurons would vary randomly with respect to one another, from stimulation to stimulation. We tested

5    this by comparing the patterns of action potential discharge of pairs of neurons between

multiple consecutive stimuli of the Schaffer collaterals. During each stimulation event,

the action potentials occurred at nearly identical times with respect to one another, and

showed an almost identical burst morphology from stimulation to stimulation. We also

checked to see whether the occurrence of action potentials between a given pair of

10   neurons during spontaneous field discharges was fixed in time. The patterns of action

potential discharges from a given pair of CA1 neurons was compared between

consecutive spontaneous field bursts during the time when the occurrence of action

potentials was clearly desynchronized. Just as in the case of stimulation-evoked action

potential discharge described above, the action potentials generated during a spontaneous

15   population discharge occurred at nearly identical times with respect to one another, and

showed a nearly identical burst morphology from one spontaneous discharge to the next.


Example 7

20   Effects of low-chloride treatment on spontaneous synaptic activity

It is possible that the anti-epileptic effects associated with chloride-cotransport

antagonism are mediated by some action on transmitter release. Blockade of chloride-

cotransport could alter the amount or timing of transmitter released from terminals, thus

affecting neuronal synchronization. To test whether low-$[Cl^-]_o$ exposure affected

25   mechanisms associated with transmitter release, intracellular CA1 responses were

recorded simultaneously with CA1 and CA3 field responses during a treatment which

dramatically increases spontaneous synaptic release of transmitter from presynaptic

terminals.

Increased spontaneous release of transmitter was induced by treatment with 4-AP

30   (100 μM). After 40 minutes exposure to 4-AP-containing medium, spontaneous

synchronized burst discharges were recorded in area CA1 and CA3. Switching to 4-AP-

containing low-$[Cl^-]_o$ medium led initially, as was shown previously, to enhanced

spontaneous bursting. High-grain intracellular recordings showed that high-amplitude

spontaneous synaptic activity was elicited by 4-AP treatment. Further exposure to low-

35   chloride medium blocked spontaneous burst discharge in CA1, although CA3 continued

to discharge spontaneously. At this time, CA1 intracellular recordings showed that

5    spontaneous synaptic noise was further increased, and remained so for prolonged
     exposure times to 4-AP-containing low-chloride medium. These data suggest that
     mechanisms responsible for synaptic release from terminals are not adversely affected by
     low-chloride exposure in a manner that could explain the blockade of 4-AP-induced
     spontaneous bursting in CA1. These results also eliminate the possibility that the effects
10   of low-$[Cl^-]o$ exposure are due to alterations in CA1 dendritic properties which would
     compromise their efficiency in conducting PSPs to the soma.


     Experimental Methods for Examples 8 to 12 .

             In all of the following experiments, $[Cl^-]o$ was reduced by equimolar replacement
15   of NaCl with $Na^+$-gluconate. Gluconate was used rather than other anion replacements
     for several reasons. First, patch-clamp studies have demonstrated that gluconate appears
     to be virtually impermeant to chloride channels, whereas other anions (including
     methylsulfate, sulfate, isethionate, and acetate) are permeable to varying degrees.
     Second, transport of extracellular potassium through glial $Na^+$, $K^+$, 2Cl cotransport is
20   blocked when extracellular chloride is replaced by gluconate but is not completely
     blocked when replaced by isethionate. Since this furosemide-sensitive cotransporter plays
     a significant role in cell swelling and volume changes of the extracellular space (ECS),
     we wished to use the appropriate anion replacement so that the effects of our treatment
     would be comparable to previous furosemide experiments. [Hochman, D.W., Baraban, S.
25   C., Owens, J. W. M., and Schwartzkroin, P. A., "Dissociation of synchronization and
     excitability in furosemide blockade of epileptiform activity." *Science*, Vol. 270, pp. 99-
     102 (1995), US Patent No. 5,902,732] Third, formate, acetate, and proprionate generate
     weak acids when employed as $Cl^-$ substitutes and lead to a prompt fall in intracellular pH;
     gluconate remains extracellular and has not been reported to induce intracellular pH
30   shifts. Fourth, for purposes of comparison we wished to use the same anion replacement
     that had been used in previous studies examining the effects of low-$[Cl^-]o$ on activity-
     evoked changes of the ECS.

             There is some suggestion that certain anion-replacements might chelate calcium.
     Although subsequent work has failed to demonstrate any significant ability of anion-
35   substitutes to chelate calcium, there is still some concern in the literature regarding this

5    issue. Calcium chelation did not appear to be an issue in the following experiments, since

resting membrane potentials remained normal and   synaptic responses (indeed,

hyperexcitable synaptic responses) could be elicited even after several hours of exposure

to medium in which $[Cl^-]_o$ had been reduced by gluconate substitution. Further, we

confirmed that calcium concentration in our low-$[Cl^-]_o$-medium was identical to that in

10   our control-medium by measurements made with $Ca2^{++}$ - selective microelectrodes.

Sprague-Dawley adult rats were prepared as previously described. Briefly,

transverse hippocampal slices, 400 μm thick, were cut using a vibrating cutter. Slices

typically contained the entire hippocampus and subiculum. After cutting, slices were

stored in an oxygenated holding chamber for at least one hour prior to recording. All

15   recordings were acquired in an interface type chamber with oxygenated (95%O2/5%CO2)

artificial cerebral spinal fluid (ACSF) at 34°-35° C. Normal ACSF contained (in

mmol/l): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 1.2 MgSO4, 26 NaHCO3, 2 CaCl2, and 10

dextrose. In some experiments, normal or low-chloride medium was used containing

bicuculline (20 μM), 4-AP (100 μM), or high-$K^+$ (12 mM). Low-chloride solutions (7,

20   16, and 21 mM $[Cl^-]_o$) were prepared by equimolar replacement of  NaCl with $Na^+$-

gluconate (Sigma Chemical Co., St. Louis, MO). All solutions were prepared so that they

had a pH of approximately 7.4 and an osmolarity of  290-300 mOsm at 35°C and at

equilibrium from carboxygenation with 95%O2 / 5%CO2.

Sharp-electrodes filled with 4 M potassium acetate were used for intracellular

25   recordings from CA1 pyramidal cells. Field recordings from the CA1 or CA3 cell body

layers were acquired with low-resistance glass electrodes filled with NaCl (2 M). For

stimulation of the Schaffer collateral pathway, a small monopolar electrode was placed on

the surface of the slice midway between areas CA1 and CA3. Spontaneous and

stimulation-evoked activities from field and intracellular recordings were digitized

30   (Neurocorder, Neurodata Instruments, New York, NY), and stored on video tape.

AxoScope software (Axon Instruments Inc.) on a PC-computer was used for off-line

analyses of data.

Ion-selective microelectrodes were fabricated according to standard methods well

known in the art. Double-barreled pipettes were pulled and broken to a tip diameter of

35   approximately 3.0 μm. The reference barrel was filled with ACSF  and the other barrel

was sylanized and the tip back-filled with a resin selective for $K^+$ (Corning 477317). The

56

5      remainder of the sylanized barrel was filled with KCl (140 mM). Each barrel was led, via

Ag/AgCl wires, to a high impedance dual-differential amplifier (WPI FD223). Each ion-

selective microelectrode was calibrated by the use of solutions of known ionic

composition and was considered suitable if it was characterized by a near-Nemstian slope

response and if it remained stable throughout the duration of the experiment.

·10         After placement in the interface chamber, slices were superfused at approximately

1 ml/minute. At this flow-rate, it took approximately 8-10 minutes for changes in

perfusion media to be completed. All of the times reported here have taken this time-

delay into account and have an error of approximately ± 2 minutes.


15                                    Example 8

                     Effects of low-[Cl-]o on CA1 field recordings

            Other studies have shown that prolonged exposure of cortical and hippocampal

slices to low-[Cl⁻]o does not affect basic intrinsic and synaptic properties such as input

resistance, resting membrane potential, depolarization-induced action-potential

20     generation, or excitatory synaptic transmission. A previous study has also partly

characterized the epileptogenic properties of low-[Cl⁻]o exposure to CA1 area of

hippocampus. The following studies were performed to observe the times of onset and

possible cessation of low-[Cl⁻]o-induced hyperexcitability and hypersynchronization.

Slices (n = 6) were initially perfused with normal medium until stable intracellular and

25     field recordings were established in a CA1 pyramidal cell and the CA1 cell body layer,

respectively. In two experiments, the same cell was held throughout the entire length of

the experiment (greater than 2 hours). In the remaining experiments (n = 4), the initial

intracellular recording was lost during the sequence of medium changes and additional

recordings were acquired from different cells. Patterns of neuronal activity in these

30     experiments were identical to those seen when a single cell was observed.

            The field and intracellular electrodes were always placed in close proximity to one

another (< 200 μm). In each case, after approximately 15-20 minutes exposure to the

low-[Cl⁻]o-medium (7 mM), spontaneous bursting developed, first at the cellular level,

and then in the field. This spontaneous field activity, representing synchronized burst

35     discharge in a large population of neurons, lasted from 5 - 10 minutes, after which time

the field recording became silent. When the field first became silent, the cell continued to

discharge spontaneously. This result suggests that population activity has been "desynchronized" while the ability of individual cells to discharge has not been impaired. After approximately 30 minutes exposure to low-[Cl⁻]o-medium, intracellular recording showed that cells continued to discharge spontaneously even though the field remained silent. The response of the cell to intracellular current injection at two time points demonstrated that the cell's ability to generate action potentials had not been impaired by low-[Cl⁻]o exposure. Further, electrical stimulation in CA1 stratum radiatum elicited burst discharges, indicating that a hyperexcitable state was maintained in the tissue.

## Example 9

### Effects of low-[Cl-]o on high-[K+]o-induced epileptiform activity in CA1

The previous set of experiments showed that tissue exposure to low-[Cl⁻]o medium induced a brief period of spontaneous field potential bursting which ceased within 10 minutes. If a reduction of [Cl⁻]o is indeed eventually capable of blocking spontaneous epileptiform (i.e. synchronized) bursting, then these results suggest that anti-epileptic effects would likely be observable only after this initial period of bursting activity has ceased. We therefore examined the temporal effects of low-[Cl⁻]o-treatment on high-[K⁺]o-induced bursting activity. Slices (n = 12) were exposed to medium in which [K⁺]o had been increased to 12 mM, and field potentials were recorded with a field electrode in the CA1 cell body layer. Spontaneous field potential bursting was observed for at least 20 minutes, and then the slices were exposed to medium in which [K⁺]o was maintained at 12 mM, but [Cl⁻]o was reduced to 21 mM. Within 15-20 minutes after the tissue was exposed to the low-[Cl⁻]o/high-[K⁺]o-medium, the burst amplitude increased and each field event had a longer duration. After a brief period of this facilitated field activity (lasting 5-10 minutes), the bursting stopped. To test whether this blockade was reversible, after at least 10 minutes of field potential silence, we switched back to high-[K⁺]o-medium with normal [Cl⁻]o. The bursting returned within 20-40 minutes. Throughout each experiment, the CA1 field response to Schaffer collateral stimulation was monitored. The largest field responses were recorded just before the cessation of spontaneous bursting, during the period when the spontaneous bursts had the largest amplitude. Even after the blockade of spontaneous bursting, however, multiple

5    population spikes were elicited by Schaffer collateral stimulation, indicating that synaptic

transmission was intact, and that the tissue remained hyperexcitable.

In four slices, intracellular recordings from CA1 pyramidal cells were acquired

along with the CA1 field recording. During the period of high-$[K^+]_o$-induced

spontaneous bursting, hyperpolarizing current was injected into the cell so that

10   postsynaptic potentials (PSPs) could be better observed. After low-$[Cl^-]_o$-blockade of

spontaneous bursting, spontaneously occurring action potentials and PSPs were still

observed. These observations further support the view that synaptic activity, per se, was

not blocked by the low-$[Cl^-]_o$ treatment.

15                                          Example 10

Low-$[Cl^-]_o$ - blockade of epileptiform activity induced by 4-AP, high-$[K^+]_o$, and

bicuculline in CA1 and CA3

We next tested whether low-$[Cl^-]_o$ treatment could block epileptiform activity in

20   areas CA1 and CA3, which was elicited by different pharmacological treatments, as we

had shown for furosemide treatment. For this set of experiments, we chose to test the

effects of low-$[Cl^-]_o$ treatment on spontaneous bursting which had been induced by high-

$[K^+]_o$ (12 mM) (n = 5), 4-AP (100 μM) (n = 4), and bicuculline (20 and 100 μM) (n = 5).

In each set of experiments, field responses were recorded simultaneously from areas CA1

25   and CA3, and in each case, the spontaneous epileptiform activity in both areas CA1 and

CA3, was reversibly blocked within 30 minutes after $[Cl^-]_o$ in the perfusion medium had

been reduced to 21 mM. These data suggest that, like furosemide, low-$[Cl^-]_o$ reversibly

blocks spontaneous bursting in several of the most commonly studied *in vitro* models of

epileptiform activity.

30

Example 11

Comparison between low-$[Cl^-]_o$ and furosemide on blockade of high-$[K^+]_o$-induced

epileptiform activity

35   The data from the previous sets of experiments are consistent with the hypothesis

that the anti-epileptic effects of both low-$[Cl^-]_o$ and furosemide are mediated by their

5      actions on the same physiological mechanisms. To further test this hypothesis, we compared the temporal sequence of effects of low-$[Cl^-]_o$ (n = 12) and furosemide (2.5 and 5 mM) (n = 4) on high-$[K+]_o$-induced bursting, as recorded with a field electrode in CA1. We found that both low-$[Cl^-]_o$ and furosemide treatment induced a similar temporal sequence of effects: an initial brief period of increased amplitude of field activity, and
10     then blockade (reversible) of spontaneous field activity. In both cases, electrical stimulation of the Schaffer collaterals elicited hyperexcited responses even after the spontaneous bursting had been blocked.

5

## Example 12
### Consequences of prolonged exposure to low-[Cl-]o medium with varied [K+]o

10    In the preceding experiments, we monitored field activity in some slices for > 1 hour after the spontaneous bursting had been blocked by low-[Cl⁻]o exposure. After such prolonged low-[Cl⁻]o exposure, spontaneous, long-lasting, depolarizing shifts developed. The morphology and frequency of these late-occurring field events appeared to be related to the extracellular potassium and chloride concentrations. Motivated by these observations, we performed a set of experiments in which we systematically varied [Cl⁻]o

15    and [K⁺]o and observed the effects of these ion changes on the late-occurring spontaneous field events.

In our first set of experiments, slices were exposed to medium containing low-[Cl]o (7 mM) and normal-[K⁺]o (3 mM) (n = 6). After 50-70 minutes exposure to this medium, spontaneous events were recorded in area CA1; these events appeared as 5-10

20    mV negative shifts in the DC field, with the first episode lasting for 30-60 seconds. Each subsequent episode was longer than the previous one. This observation suggested that ion-homeostatic mechanisms were diminished over time as a result of the ion concentrations in the bathing medium. In some experiments (n = 2) in which these negative DC field shifts had been induced, intracellular recordings from CA1 pyramidal

25    cells were acquired simultaneously with the CA1 field recordings.

For these experiments, the intracellular and field recordings were acquired close to one another (< 200 μm). Prior to each negative field shift (10-20 seconds), the neuron began to depolarize. Cellular depolarization was indicated by a decrease in resting membrane potential, an increase in spontaneous firing frequency, and a reduction of

30    action potential amplitude. Coincident with the onset of the negative field shifts, the cells became sufficiently depolarized so that they were unable to fire spontaneous or current-elicited (not shown) action potentials. Since neuronal depolarization began 10-20 seconds prior to the field shift, it may be that a gradual increase in extracellular potassium resulted in the depolarization of a neuronal population, thus initiating these field events.

35    Such an increase in [K⁺]o might be due to alterations of the chloride-dependent glial cotransport mechanisms that normally move potassium from extracellular to intracellular

spaces. To test whether increases in $[K^+]o$ preceded these negative field shifts (and paralleled cellular depolarization), experiments (n=2) were performed in which a $K^+$-selective microelectrode was used to record changes in $[K^+]o$.

In each experiment, the $K^+$-selective microelectrode and a field electrode were placed in the CA1 pyramidal layer close to one another (< 200 μm), and a stimulation pulse was delivered to the Schaffer collaterals every 20 seconds so that the magnitude of the population spike could be monitored. Multiple spontaneously occurring negative field shifts were evoked by perfusion with low-$[Cl^-o]$ (7 mM) medium. Each event was associated with a significant increase in $[K^+]o$ , with the $[K^+]o$ increase starting several seconds prior to the onset of negative field shift. A slow 1.5-2.0 mM increase in $[K^+]o$ occurred over a time interval of approximately 1-2 minute seconds prior to the onset of each event. The stimulation-evoked field responses slowly increased in amplitude over time, along with the increasing $[K^+]o$, until just before the negative field shift.

In a second set of experiments (n = 4), $[K^+]o$ was increased to 12 mM and $[Cl-]o$ was increased to 16 mM. After 50 -90 minutes exposure to this medium, slow oscillations were recorded in area CA1. These oscillations were characterized by 5-10 mV negative DC shifts in the field potential and had a periodicity of approximately 1 cycle/40 seconds. Initially, these oscillations occurred intermittently and had an irregular morphology. Over time, these oscillations became continuous and developed a regular waveform. Upon exposure to furosemide (2.5 mM), the amplitude of the oscillations was gradually decreased and the frequency increased until the oscillations were completely blocked. Such low-$[Cl]o$ - induced oscillations in tissue slices have not been previously reported. However, the temporal characteristics of the oscillatory events bear a striking resemblance to the low-$[Cl]o$ - induced $[K^+]o$ oscillations which were previously described in a purely axonal preparation.

In a third set of experiments (n = 5) $[Cl^-]o$ was further increased to 21 mM and $[K^+]o$ was reduced back to 3 mM. In these experiments, single, infrequently occurring negative shifts of the field potential developed within 40 - 70 minutes (data not shown). These events (5-10 mV) lasting 40-60 seconds, occurred at random intervals, and maintained a relatively constant duration throughout the experiment. These events could sometimes be elicited by a single electrical stimulus delivered to the Schaffer collaterals.

Finally, in a final set of experiments (n = 5), $[Cl^-]o$ was kept at 21 mM and $[K^+]o$ was raised to 12 mM. In these experiments, late-occurring spontaneous field events were not observed during the course of the experiments (2-3 hours).


## Example 13

## Changes in $[K^+]_0$ during low-chloride exposure


Sprague-Dawley adult rats were prepared as previously described. Transverse hippocampal slices, 400 μm thick, were cut with a vibrating cuter and stored in an oxygenated holding chamber for 1 hour before recording. A submersion-type chamber was used for $K^+$-selective microelectrode recordings. Slices were perfused with oxygenated (95% $O_2$ – 5% $CO_2$) artificial cerebrospinal fluid (ACSF) at 34-35° C. Normal ACSF contained 10 mM dextrose, 124 mM NaCl 3 mM KCl, 1.25 mM $NaH_2PO_4$, 1.2 mM $MgSO_4$, 26 mM $NaHCO_3$ and 2 mM $CaCl_2$. In some experiments, normal or low-chloride medium was used containing 4-aminopyridine (4-AP) at 100μM. Low-chloride solutions (21 mM $[Cl]_0$) were prepared by equimolar replacement of NaCl with Na+-gluconate (Sigma Chemical Co.).

Field recordings from the CA1 or CA3 cell body layers were acquired with low-resistance glass electrodes filled with NaCl (2M). For stimulation of the Schaffer collateral pathway, a monopolar stainless-steel electrode was placed on the surface of the slide midway between areas CA1 and CA3. All recordings were digitized (Neurorocorder, Neurodata Instruments, New York, NY) and stored on videotape.

$K^+$ selective microelectrodes were fabricated according to standard methods. Briefly, the reference barrel of a double-barreled pipette was filled with ACSF, and the other barrel was sylanized and the tip back-filled with KCl with $K^+$-selective resin (Corning 477317). Ion-selective microelectrodes were calibrated and considered suitable if they had a Nernstian slope response and remained stable throughout the duration of the experiment.

Exposure of hippocampal slices to low-$[Cl-]_0$ medium has been shown to include a temporally-dependent sequence of changes on the activity of CA1 pyramidal cells, with three characteristics phases, as described above. In brief, exposure to low-$[Cl-]_0$ medium results in a brief period of increased hyperexcitability and spontaneous epileptiform

discharge. With further exposure to low-$[Cl^-]_0$ medium, spontaneous epileptiform activity is blocked, but cellular hyperexcitability remains, and action potential firing times become less synchronized with one another. Lastly, with prolonged exposure, the action potential firing times become sufficiently desynchronized so that stimulation-evoked field responses completely disappear, yet individual cells continue to show monosynapticlly-evoked responses to Schaffer collateral stimulation. The following results demonstrate that the antiepileptic effects of furosemide on chloride-cotransport antagonism are independent of direct actions on excitatory synaptic transmission, and are a consequence of a desynchronization of population activity with our any associated decrease in excitability.

In six hippocampal slices, $K^+$-selective and field microelectrodes were placed in the CA1 cell body layer, and a stimulating electrode was placed on the Schaffer collateral pathway, and single-pulse stimuli (300 μs) were delivered every 20 seconds (Fig. 19). After stable baseline $[K^+]_0$ was observed for at least 20 minutes, the perfusion was switched to low-$[Cl^-]_0$ medium. Within 1-2 minutes of exposure to low-$[Cl^-]_0$ medium, the field responses became hyperexcitable as the $[K^+]_0$ began to rise. After approximately 4-5 minutes of exposure to low-$[Cl^-]_0$ medium the magnitude of the field response diminished, until it was completely abolished. The corresponding recording of $[K^+]_0$) showed that potassium began to rise immediately after exposure to low-$[Cl^-]_0$ medium, and that the peak of this $[K^+]_0$ rise corresponded in time to the maximally hyperexcitable CA1 field response. Coincident with the reduction of the magnitude of the field response, the $[K^+]_0$ began to diminish until after 8-10 minutes exposure to low-$[Cl^-]_0$ medium, it became constant for the remainder of the experiment at 1.8-2.5 mM above control levels. Four slices were switched back to control medium and allowed to fully recover. The experiment was then repeated with the $K^+$-selective microelectrode placed in the stratum radiatum. A similar sequence of changes in $[K^+]_0$ was observed in the dendritic layer, with the values of $[K^+]_0$ being 0.2-0.3 mM less than those observed in the cell body layers.

In four hippocampal slices, the responses of stimulation-evoked changes in $[K^+]_0$ between control conditions and after the CA1 field response was completely abolished by low-$[Cl^-]_0$ exposure were compared. In each slice, the $[K^+]_0$-selective measurements were acquired first in the cell body layer, and then after allowance for complete recovery in

5    control medium, the experiment was repeated with the $K^+$-selective electrode moved to

the stratum radiatum. Each stimulation trial consisted of a 10 Hz volley delivered to the

Schaffer collateral for 5 seconds. The peak rises in $[K^+]_0$ were similar between control

conditions an after prolonged exposure to low-$[Cl^-]_0$ medium, and between the cell body

and dendritic layers. However, the recovery times observed after prolonged exposure to

10   low-$[Cl^-]_0$ were significantly longer than those observed during control conditions.

These results demonstrate that the administration of furosemide resulted in

increased $[K^+]_0$ in the extracellular spaces. Exposure of the brain tissue to low-$[Cl^-]_0$

medium immediately induced a rise in $[K^+]_0$ by 1-2 mM, which remained throughout the

duration of exposure, and was coincident with the initial increase in excitability and the

15   eventual abolishment of the CA1 field response. This loss of CA1 field response during

low-$[Cl^-]_0$ exposure is most likely due to the desynchronization of neuronal firing times.

Significantly, the stimulation-evoked increases in $[K^+]_0$, in both the cell body and

dendritic layers were nearly identical before and after the complete low-$[Cl^-]_0$ blockade of

the CA1 field response. This data suggests that comparable stimulation-evoked synaptic

20   drive and action potential generation occurred under control conditions and after low-$[Cl^-]_0$ blockade of the field. Together these data demonstrate that the antiepileptic and

desynchronizing effects of the chloride-cotransport antagonist, furosemide, are

independent of direct actions on excitatory synaptic transmission and are a consequence

of a desynchronization of population activity without decrease in excitability.

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## Example 14

### Changes in extracellular pH during low-chloride exposure

Antagonists of the anion/chloride-dependent cotransporter, such as furosemide

30   and low-$[Cl-]_0$, may affect extracellular pH transients that might contribute to the

maintenance of synchronized population activity. Rat hippocampal brain slices were

prepared as described in Example 13, except the $NaHCO_3$ was substituted by equimolar

amount of HEPES (26 nM) and an interface-type chamber was used.

In four hippocampal brain slices continuous spontaneous bursting was elicited by

35   exposure to medium containing 100 μM 4-AP, as described in Example 13. Field

recordings were acquired simultaneously from the cell body layers in areas CA1 and

CA3. A stimulus delivered every 30 seconds to the Schaffer collaterals throughout the duration of the experiments. After at least 20 minutes of continuous bursting was observed, the slices were exposed to nominally bicarbonate free, 4-AP-containing HEPES medium. There were no significant changes observed in the spontaneous or stimulation-evoked field responses resulting from prolonged exposure (0.2 hours) to HEPES medium. After the slices had been exposed for at least 2 hours to the HEPES medium, the perfusion was switched to 4-AP-containing HEPES medium in which the $[Cl^-]_0$ had been reduced to 21 mM. Exposure to the low-$[Cl^-]_0$ HEPES medium induced the identical sequences of events, and at the same time course, as had previously been observed with low-$[Cl^-]_0$ $NaHCO_3$-containing medium. After complete blockade of spontaneous bursting, the perfusion medium was switched back HEPES medium with normal $[Cl^-]_0$. Within 20-40 minutes, spontaneous bursting resumed. At the time the spontaneous bursting had resumed, the slices had been perfused with nominally bicarbonate-free HEPES medium for greater than 3 hours.

This data suggests that the actions of chloride-cotransport antagonism on synchronization and excitability are independent of affects on the dynamics of extracellular pH.

Figure 4 illustrates a schematic model of ion cotransport under conditions of reduced $[Cl^-]$. Fig. 4A, left panel, shows that the chloride gradient necessary for the generation of IPSPs in neurons is maintained by efflux of ions through a furosemide-sensitive $K^+$, $Cl^-$ cotransporter. Under normal conditions, a high concentration of intracellular potassium (maintained by the $3Na^+$, $2K^+$-ATPase pump) serves as the driving force for the extrusion of $Cl^-$ against its concentration gradient. In glial cells, as shown in the right panel of Fig. 4A, the movement of ions through the furosemide-sensitive $Na^+$, $K^+$, $2Cl^-$ - cotransporter is from extracellular to intracellular spaces. The ion-gradients necessary for this cotransport are maintained, in part, by the "transmembrane sodium cycle": sodium ions taken into glial cells through $Na^+$, $K^+$, $2Cl^-$ - cotransport are continuously extruded by the $3Na^+$, $2K^+$,- ATPase pump sothat a low intracellular sodium concentration is maintained. The rate and direction of ion-flux through the furosemide-dependent cotransporters are functionally proportional to their ion-product differences written as $[K^+]_i$ x $[Cl^-]_i$ - $[K^+]_0$ x $[Cl^-]_0$) for neuronal $K^+$, $Cl^-$ cotransport and as $[Na^+]_i$ x $[K^+]_i$ x $[Cl^-]^2_i$ - $[Na^+]_0$ x $[K^+]_0$ x $[Cl^-]^2_0$) for glial $Na^+$, $K^+$, $2Cl^-$ cotransport. The sign of

5     these ion-product differences show the direction of ion transport with positive being from intracellular to extracellular spaces.

Figure 4B shows a schematic phenomenological model that explains the emergence of the late-occurring spontaneous field events that arise as a result of prolonged low -[Cl⁻] o exposure. We denote the ion-product differences for neurons and

10    glia as $Q_N$ and $Q_G$, respectively. Under control conditions (1), the differences of the ion-products for neurons are such that $K^+$ and Cl⁻ are cotransported from intracellular to extracellular spaces ($Q_N > 0$); the differences in ion-products for glial cells are such that $Na^+$, $K^+$ and Cl⁻ are cotransported from the ECS to intracellular compartments ($Q_G < 0$). When [Cl⁻] o is reduced (2), the ion-product differences are altered so that neuronal efflux

15    of KCl is increased; however, the glial icon cotransport is reversed ($Q_G > 0$), s that there is a net efflux of KCl and NaCl from intracellular to extracellular spaces. These changes result in buildup of extracellular potassium over time. Eventually, $[K^+]_o$ reaches a level that induces the depolarization of neuronal populations, resulting in an even larger accumulation of $[K^+]_o$. This large accumulation of extracellular ions then serves to

20    reverse the ion-product differences so that KCl is moved from extracellular to intracellular spaces ($Q_N < 0$, $Q_G < 0$) (3). Further clearance of the extracellular potassium eventually resets the transmembrance ion gradients to initial conditions. By cycling through this process, repetitive negative field events are generated.

## CLAIMS

1.    A method for treating a condition of the central nervous system in a mammalian subject comprising administering an effective amount of a treatment composition having ion-dependent cotransporter antagonist activity.

2.    A method of claim 1 for treating a condition of the central nervous system selected from one of the following: seizures; seizure disorders; epilepsy; status epilepticus; migraine headache; cortical spreading depression; intracranial hypertension; central nervous system edema; neuropsychiatric disorders; neurotoxicity; head trauma; stroke; ischemia and hypoxia.

3.    A method of claim 1, wherein the treatment composition has cation-chloride cotransporter antagonist activity.

4.    A method of claim 3, wherein the treatment composition has $Na^+K^+Cl^-$ cotransporter antagonist activity.

5.    A method of claim 1, wherein the treatment composition has higher activity as a glial cell ion-dependent cotransporter antagonist than as a neuronal cell ion-dependent cotransporter antagonist.

6.    A method of claim 1, wherein the treatment composition has higher activity as a glial cell ion-dependent cotransporter antagonist than as a renal cell ion-dependent cotransporter antagonist.

7.    A method of claim 1, wherein the subject is a human.

8.    A method for enhancing cortical function in an area of the central nervous system of a mammalian subject comprising administering an effective amount of a treatment composition having ion-dependent cotransporter agonist activity.

9.    A method of claim 8, wherein the area of the central nervous system of the subject is an area associated with at least one of cognition, learning and memory functions.

10.    A method of claim 9, wherein the treatment composition has cation-chloride cotransporter agonist activity.

11.    A method for reducing the sensation of pain in a mammalian subject comprising administering an effective amount of a treatment composition having ion-dependent cotransporter antagonist activity to the peripheral nervous system of the subject.

12.    A method of claim 11, wherein the treatment composition has cation-chloride cotransporter antagonist activity.

13.    A method of claim 11, wherein the treatment composition has $Na^+K^+Cl^-$ cotransporter antagonist activity.

14.    A method of claim 11, wherein the treatment composition has higher activity as a glial cell ion-dependent cotransporter antagonist than as a neuronal cell ion-dependent cotransporter antagonist.

15.    A method of claim 11, wherein the treatment composition has higher activity as a glial cell ion-dependent cotransporter antagonist than as a renal cell ion-dependent cotransporter antagonist.

16.    A method for treating a condition of the central nervous system comprising administering a composition that modulates at least one of the ionic concentrations in the extracellular space and the ionic gradients of the central nervous system.

17.    A method of claim 16 for treating a condition of the central nervous system selected from one of the following: seizures; seizure disorders; epilepsy; status epilepticus; migraine headache; cortical spreading depression; intracranial hypertension; central nervous system edema; neuropsychiatric disorders; neurotoxicity; head trauma; stroke; ischemia and hypoxia.

18.    A method for treating status epilepticus comprising administering an effective amount of a treatment composition having ion-dependent cotransporter activity in combination with an anti-seizure composition.

19.    A method of claim 18 comprising administering an effective amount of a loop diuretic in combination with an anti-seizure composition.

20.    A method of claim 18, wherein the anti-seizure composition is Phenobarbitol.

21.    A method of claim 18 comprising administering an effective amount of furosemide in combination with an anti-seizure composition.

22.    A method of claim 21, wherein the anti-seizure composition is Phenobarbitol.

23.    A treatment agent comprising a composition having ion-dependent cotransporter antagonist activity, and having higher activity as a glial cell ion-dependent cotransporter antagonist than as a neuronal cell ion-dependent cotransporter antagonist.

24.    A treatment agent of claim 23, wherein the composition additionally has higher activity as a glial cell ion-dependent cotransporter antagonist than as a renal cell ion-dependent cotransporter antagonist.

25.    A treatment agent of claim 23 comprising a composition having cation-chloride cotransporter antagonist activity.

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      26.     A treatment agent comprising a composition having ion-dependent
cotransporter agonist activity, and having higher activity as a glial cell ion-dependent
cotransporter agonist than as a neuronal cell ion-dependent cotransporter agonist.

10      27.     A treatment agent of claim 26, wherein the composition additionally has
higher activity as a glial cell ion-dependent cotransporter agonist than as a renal cell ion-
dependent cotransporter agonist.

      28.     A treatment agent of claim 26 comprising a composition having cation-
15    chloride cotransporter agonist activity.

      29.     A treatment agent comprising a combination of a first composition having ion-
dependent cotransporter antagonist activity and a second composition having anti-seizure
or anti-convulsant activity.

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      30.     A treatment agent of claim 29 comprising an ion-dependent cotransporter
antagonist and a second composition selected from the group consisting of: phenytoin,
carbamazepine, barbiturates, Phenobarbital, pentobarbital, mephobarbital, trimethadione,
mephenytoin, paramethadione, phenthenylate, phenacemide, metharbital,
25    benzchlorpropanmide, phensuximide, primidone, methsuximide, ethotoin,
aminoglutethimide, diazepam, clonazepam, clorazepate, fosphenytoin, ethosuximide,
valporate, felbamate, gabapentin, lamotrigine, topiramate, vigrabatrin, tiagabine,
zonisamide, clobazam, thiopental, midazoplam, propofol, levetiracetam, oxcarbazepine,
CCPene, GYK152466 and sumatriptan.

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      31.     A treatment agent of claim 29 wherein the first composition has cation-
chloride cotransporter antagonist activity.

      32.     A container containing a first composition having ion-dependent
35    cotransporter antagonist activity and a second composition having anti-seizure or anti-
convulsant activity.

33.    A method for assessing the physiological condition of a biological material, comprising:

maintaining at least one sample population of the biological material in one of the following systems: a cell culture system; a tissue culture system; an organ culture system; and an intact organism:

acquiring test data relating to one or more geometrical or optical properties of the sample population; and

comparing the test data acquired to comparison data relating to one or more geometrical or optical properties of a comparison cell population, the comparison data representing one or more geometrical or optical properties of the comparison cell population in a predetermined physiological state, whereby changes in the one or more geometrical or optical properties reflected in the test data compared to the comparison data represent changes in the physiological state of the sample population.

34.    A method of claim 33, additionally comprising acquiring multiple test data sets relating to one or more geometrical or optical properties at multiple, predetermined spatial locations in the sample population.

35.    A method of claim 33, additionally comprising exposing the sample population to a physiological challenge prior to acquiring the test data.

36.    A method of claim 35, additionally comprising acquiring control data relating to the one or more geometrical properties of the sample population prior to exposing the sample population to a physiological challenge.

37.    A method of claim 36, additionally comprising comparing the test data to the control data to assess changes in the one or more geometrical or optical properties of the sample population representing changes in the physiological state of the sample population.

38.    A method of claim 33, wherein the comparison data is derived from empirically determined controls.

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39. A method of claim 35, wherein the physiological challenge is selected from the group consisting of: exposure to a test agent, a test condition, a drug, a hormone, a biological agent, a toxin, an infectious agent, radiation, chemotherapy, deprivation of a metabolite or nutrient, electrical stimulation, inflammatory agent, oncogen.

40. A method of claim 33, additionally comprising maintaining multiple sample populations in an *in vitro* culture system.

41. A method of claim 33, wherein the test data acquired relates to one or more optical properties selected from the group consisting of: reflection, refraction, diffraction, absorption, scattering, birefringence, refractive index and Kerr effect.

42. A method according to claim 33, wherein the biological material is a viable sample population maintained in a cell culture system.

43. A method according to claim 33, wherein the biological material is a viable, intact organism.

44. A system for assessing the physiological condition of a biological material, comprising:

a platform for supporting an optically transparent container of biological material;

an illumination source for illuminating the biological material;

an optical detector for acquiring test data relating to a geometrical or optical property of the biological material; and

a data storage and analysis device for storing data relating to a geometrical or optical property of the biological material, and comparing test data with a control data profile to generate a comparison data set relating to geometrical or optical property of the biological material;

45.     A system of claim 44, wherein the platform is an automated stage operated by a control device capable of locating and moving the stage to predetermined x-y coordinates.

46.     A system of claim 44, additionally comprising a data output device capable of displaying the comparison data in a visual format.

47.     A system of claim 46, wherein the data output device is capable of displaying comparison data in a graphical or image format.

48.     A system of claim 44, wherein the optical detector is a charge coupled device (CCD).

49.     A method for identifying optical contrast enhancing agents useful for enhancing the sensitivity of optical detection of a biological material comprising:

maintaining at least one sample population of the biological material in one of the following systems: a cell culture system; a tissue culture system; an organ culture system; and an intact organism;

exposing the at least one sample population to a candidate optical contrast enhancing agent;

acquiring test data relating to one or more optical properties of the sample population subsequent to exposure to the candidate optical contrast enhancing agent; and

comparing the test data acquired to comparison data relating to the one or more optical properties of the sample population.

50.     A method for screening a candidate compound for activity in treating or preventing a central nervous system disorder, comprising:

preparing a central nervous system cell sample under conditions that preserve the viability of cells in the cell sample;

introducing an agent to the cell sample that is capable of changing at least one of the ionic concentration and the ionic gradient in the cell sample without killing cells;

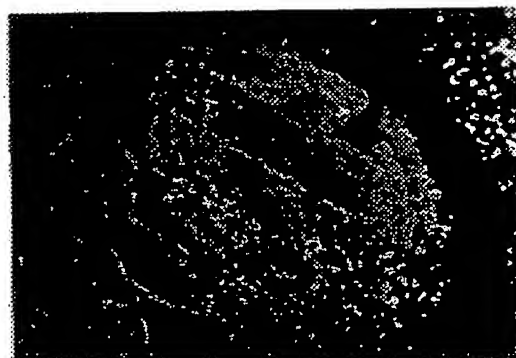determining and recording dimensions of cells in the cell sample following introduction of the agent using an optical detection technique;

introducing a candidate compound to the cell sample;

determining and recording dimensions of cells in the cell sample following introduction of the candidate compound using an optical detection technique; and

comparing the dimensions of cells following introduction of the agent with the dimensions of cells following introduction of the candidate compound, whereby changes in the dimensions of cells are related to changes in the ionic balance of the cell sample.

51.    A method for screening a candidate compound for activity in treating or preventing a central nervous system disorder, comprising:

preparing a central nervous system cell sample under conditions that preserve the viability of cells in the cell sample;

introducing an agent to the cell sample that is capable of changing at least one of the ionic concentration and the ionic gradient in the cell sample without killing cells;

determining and recording intrinsic optical properties of cells in the cell sample following introduction of the agent using an optical detection technique;

introducing a candidate compound to the cell sample;

determining and recording intrinsic optical properties of cells in the cell sample following introduction of the candidate compound using an optical detection technique; and

comparing the intrinsic optical properties of cells following introduction of the agent with the intrinsic optical properties of cells following introduction of the candidate compound.

52.    A method for screening a candidate compound for activity as an ion-dependent cotransporter agonist or antagonist, comprising:

introducing an agent to a cell sample that is capable of changing at least one of the ionic concentration and the ionic balance in a cell sample without killing cells;

observing and recording at least one property of the cell sample that is representative of at least one of the ionic concentration and the ionic balance in the cell sample following introduction of the agent;

5        introducing a candidate compound to the cell sample;

observing and recording at least one property of the cell sample that is representative of at least one of the ionic concentration and the ionic balance in the cell sample following introduction of the candidate compound; and

comparing the at least one property of the cell sample recorded following

10    introduction of the agent with the at least one property of the cell sample recorded following introduction of the candidate compound, and thereby determining changes in at least one of the ionic concentration and the ionic balance in the cell sample.
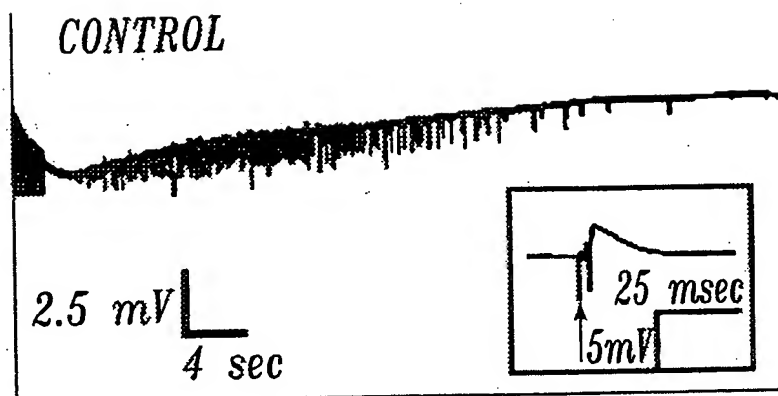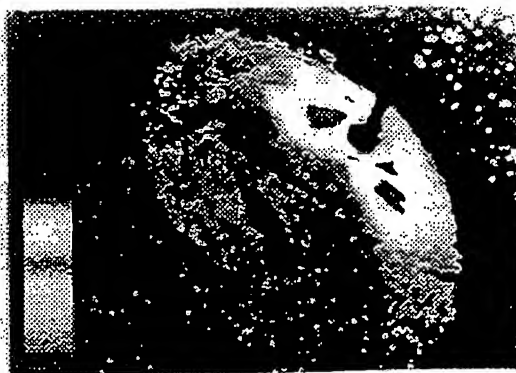
Fig. 1A



Fig. 1A1

2.5 mM FUROSEMIDE

Fig. 1B

Fig. 1B1

Fig. 1C



Fig. 1C1



Fig. 1D
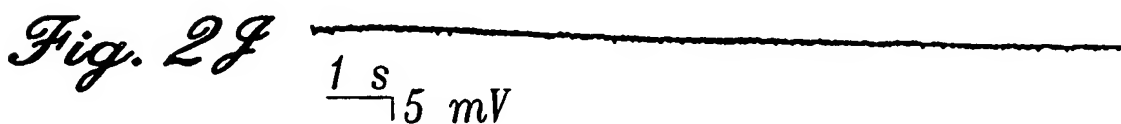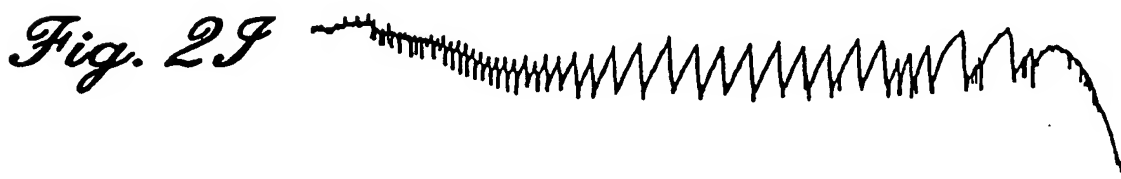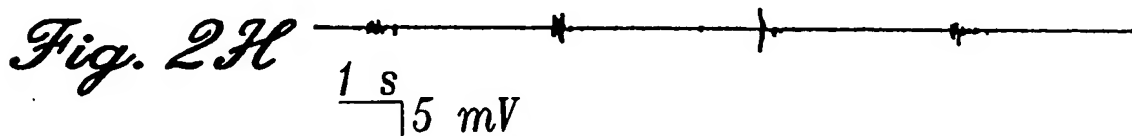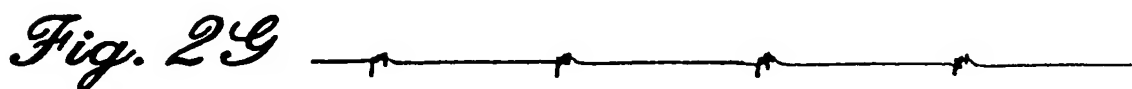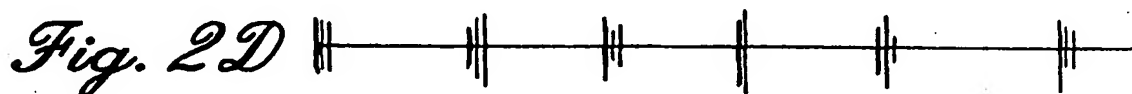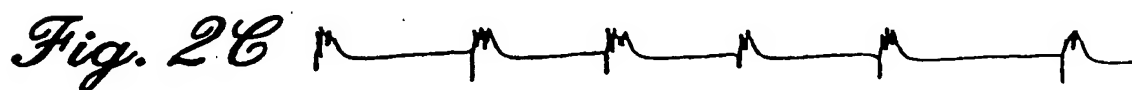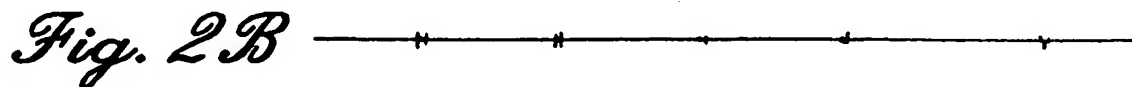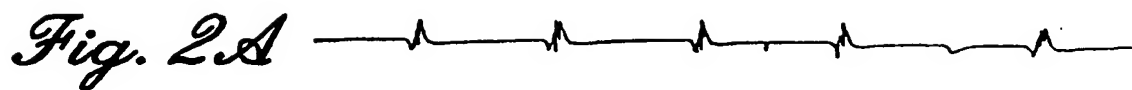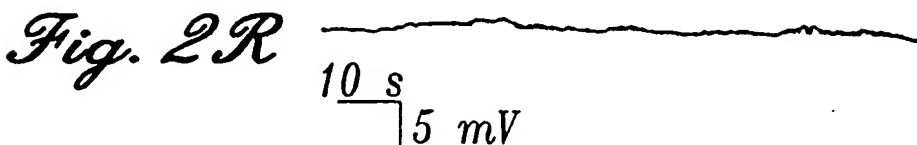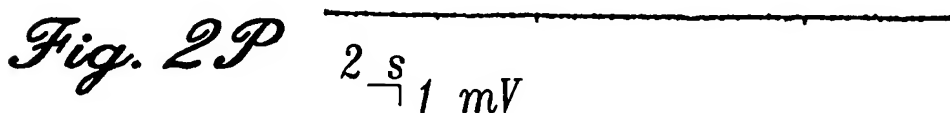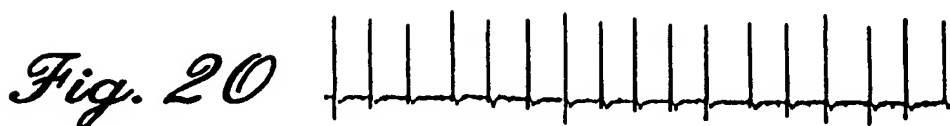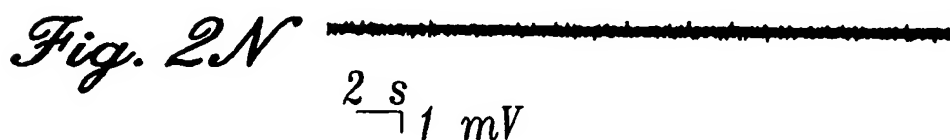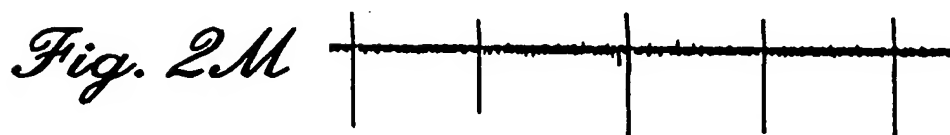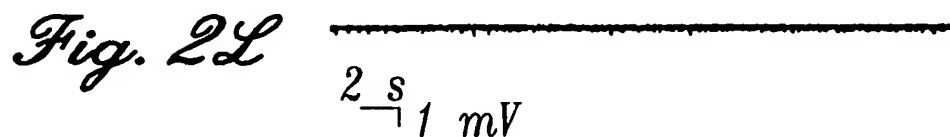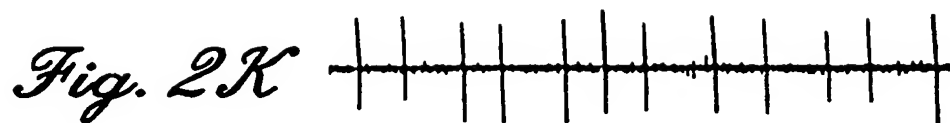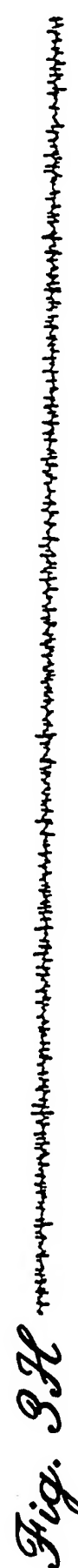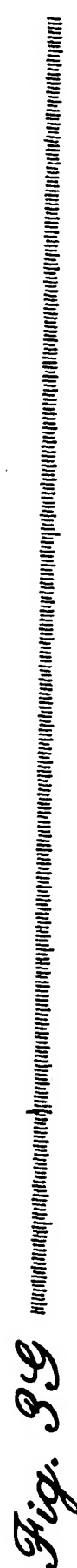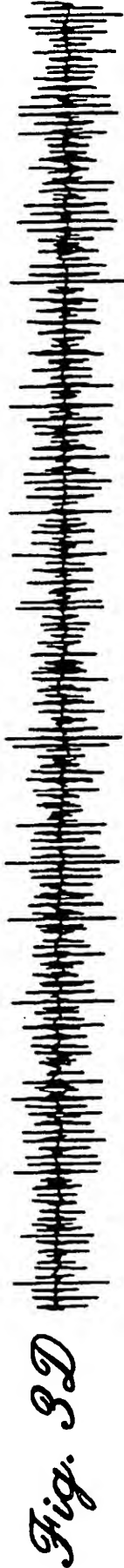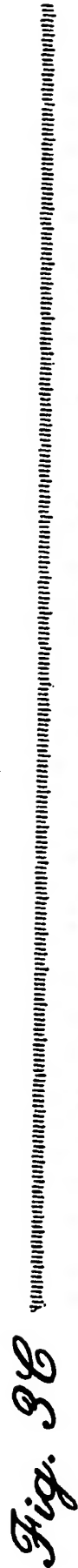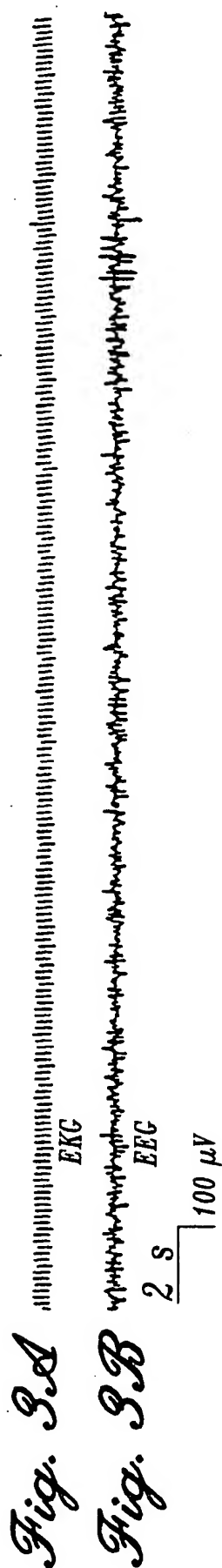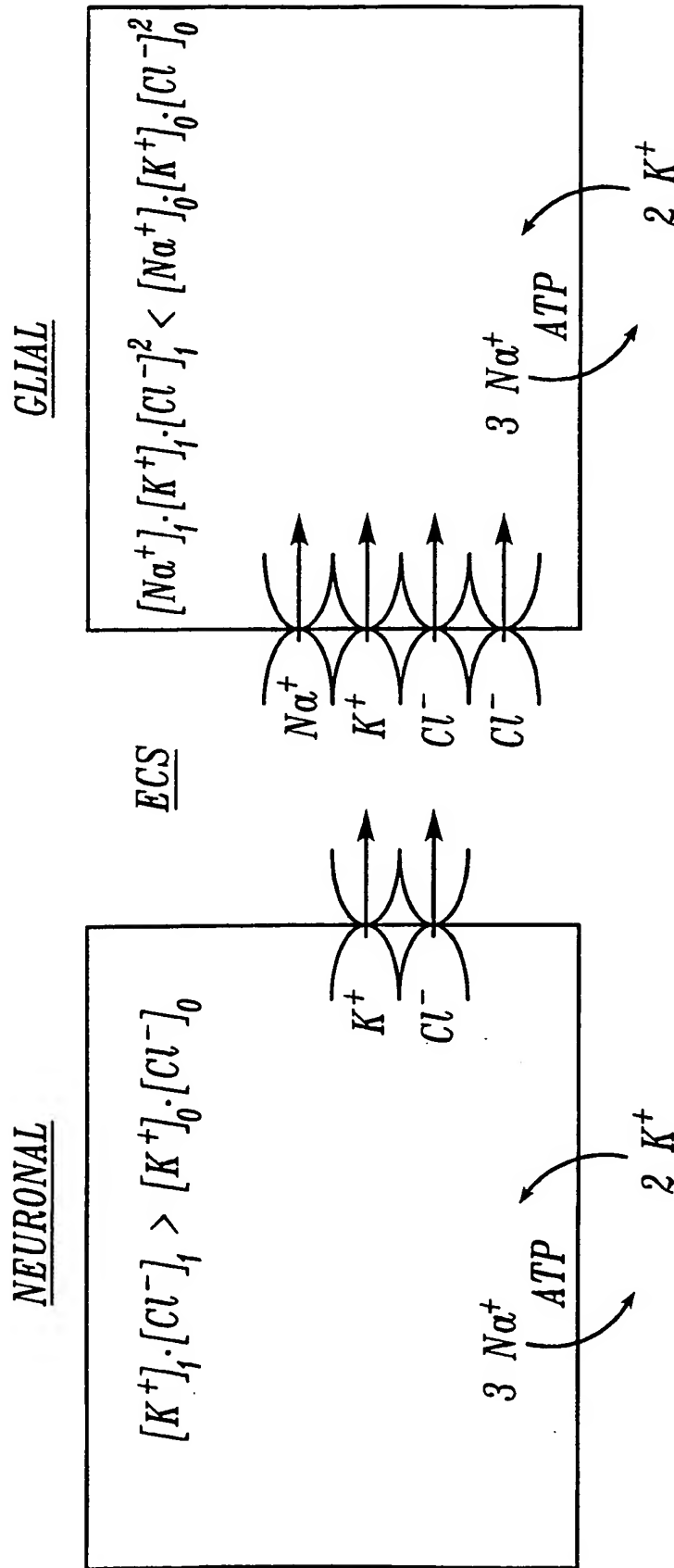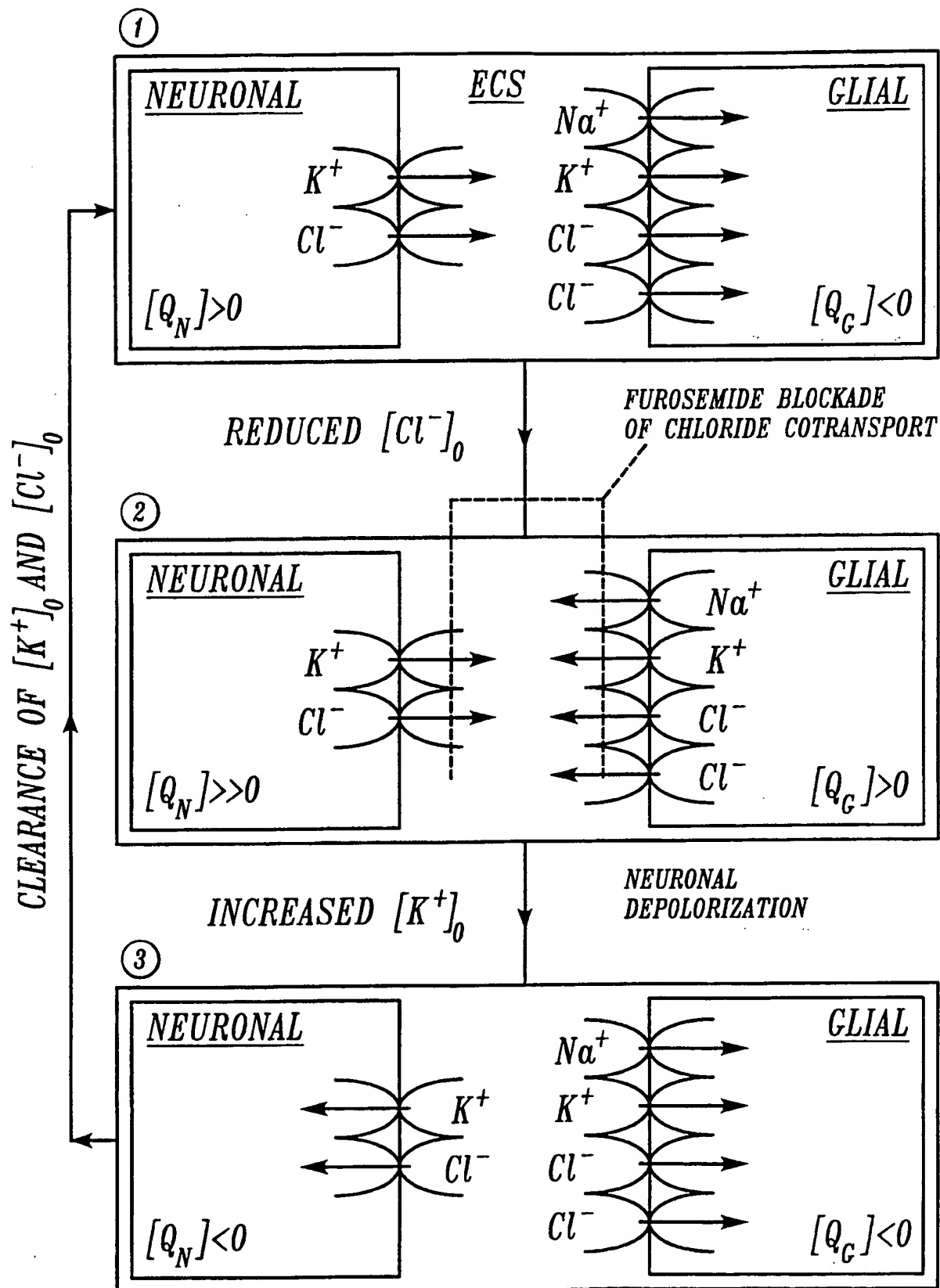
*Fig. 2A*

*Fig. 2B*

*Fig. 2C*

*Fig. 2D*

*Fig. 2E*

*Fig. 2F*

*Fig. 2G*

*Fig. 2H*

1 s

5 mV

*Fig. 2I*

*Fig. 2J*

1 s

5 mV

5/8

Fig. 2K

Fig. 2L

2 s
1 mV

Fig. 2M

Fig. 2N

2 s
1 mV

Fig. 2O

Fig. 2P

2 s
1 mV

Fig. 2Q

Fig. 2R

10 s
5 mV

Fig. 3A

Fig. 3B

EKG

EEG

2 s

100 µV

Fig. 3C

Fig. 3D

Fig. 3E

Fig. 3F

Fig. 3G

Fig. 3H

*Fig. 4A*

*Fig. 4B*

# INTERNATIONAL SEARCH REPORT

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7)   :C12N 9/00; A61K 31/44
US CL   : 514/347, 870

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S.  :   514/347, 870

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 3,676,454 A (VIDA) 11 JULY 1972, column 1, lines 9-23. | 18-22 and 29-32 |
| Y | US 4,154,652 A (SAWAMURA et al.) 15 MAY 1979, column 2, lines 18-56 and column 4, lines 37-42. | 33-43 |
| X --- Y | US 5,486,530 A (BOELKE et al.) 23 JANUARY 1996, column 1, lines 6-46 and column 2, lines 17-41. | 1-7 and 23-25 ----------- 18-22 and 29-43 |
| X --- Y | US 5,498,519 A (RUBIN et al.) 12 MARCH 1996, column 1, lines 58-67, column 2, lines 17-28, and column 3, lines 46-67. | 1-7 and 23-25 ----------- 33-43 |

[X] Further documents are listed in the continuation of Box C.      [ ]      See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | | |
| "E" | earlier document published on or after the international filing date | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | | |
| "O" | document referring to an oral disclosure, use, exhibition or other means | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "P" | document published prior to the international filing date but later than the priority date claimed | "&" | document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 MARCH 2000 | 1 4 APR 2000 |

| Name and mailing address of the ISA/US | Authorized officer |
|---|---|
| Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 | MARJORIE MORAN |
| Facsimile No.   (703) 305-3230 | Telephone No.   (703) 308-1235 |

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT ||| 
| X | US 5,571,842 A (KLEEMANN et al.) 05 NOVEMBER 1996, col. 4, lines 36-61 and col. 5, lines 8-28. | 1-7 and 23-25 |
| X | US 5,834,466 A (RAMASAMY et al.) 10 NOVEMBER 1998, column 3, lines 24-35 and column 9, line 45-column 6, line 3. | 1-7 and 23-25 |
| X, P ----- Y, P | US 5,902,732 A (HOCHMAN) 11 MAY 1999, column 2, lines 6-33, column 3, line 34-column 5, line 15, column 7, lines 44-67, column 10, lines 29-36, and column 19, line 34-column 20, line 7. | 1-7, 23-25, 33-43 ---------- 18-22, 29-32 |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/30806

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**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
   1-7, 18-25, and 29-43

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

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**Remark on Protest**    ☐ The additional search fees were accompanied by the applicant's protest.

                          ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)*

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EAST (USPAT, JPO, EPO, DERWENT), BIOSIS: terms: epilipsy, status adj elipticus, seizure, ischemia, furosemide, bumetanide, ethacrinic, ethacrynic, phenobarbitol, sodium or potassium or chloride (near2) channel

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-7, drawn to a method for treating a condition of the central nervous system (CNS) by administering a compound having ion-dependent cotransporter antagonist activity.
Group II, claims 8-10, drawn to a method for enhancing cortical function by administering a compound having ion-dependent cotransporter agonist activity.
Group III, claims 11-15, drawn to a method for reducing pain by administering a compound having ion-dependent cotransporter antagonist activity.
Group IV, claims 16-17, drawn to a method for treating a condition of the central nervous system (CNS) by administering a compound which modulates ionic concentrations/gradients.
Group V, claims 18-22, drawn to a method for treating status elipticus by administering a compound having ion-dependent cotransporter activity.
Group VI, claims 23-25, drawn to a treatment agent comprising a compound having ion-dependent cotransporter antagonist activity.
Group VII, claims 26-28, drawn to a treatment agent comprising a compound having ion-dependent cotransporter agonist activity.
Group VIII, claims 29-32, drawn to a treatment agent comprising a compound having ion-dependent cotransporter antagonist activity and a second composition having anti-seizure or anticonvulsant activity.
Group IX, claims 33-43, drawn to a method for assessing the physiological condition of a biological material.
Group X, claims 44-48, drawn to a system (device) for assessing the physiological condition of a biological material.
Group XI, claim 49, drawn to a method for identifying optical contract enhancing agents.
Group XII, claim 50, drawn to a method for screening a compound for activity in treatment or prevention of a CNS disorder by measuring changes in cell dimensions.
Group XIII, claim 51, drawn to a method for screening a compound for activity in treatment or prevention of a CNS disorder by measuring changes in optical properties.
Group XIV, claim 52, drawn to a method for screening a compound for activity in treatment or prevention of a CNS disorder by measuring changes in ionic concentrations.

The inventions listed as Groups I to VIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: compounds having ion-dependent cotransporter antagonist and agonist activity are known in the art, as are methods of treating disorders of the CNS by administering those compounds, therefore the agents and methods of treatment recited in Groups I to VIII lack a special technical feature.

The inventions listed as Groups IX to XIV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: methods of measuring various cell and tissue parameters (size, optical properties, ionic flux, etc.) are known in the art, therefore there is no special technical feature linking the methods of Groups IX-XIV.

In addition, there is no linking concept between the methods of Groups I-V (methods of treatment) and the methods of Groups IX-XIV (methods for assessing).